PART TWO

CHAPTER THIRTEEN

Small Ermine Moths

Role of Pheromones in Reproductive Isolation and Speciation

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Introduction

Small ermine moths belong to the genus *Yponomeuta* (Yponomeutidae) that comprises about 75 species distributed globally but mainly in the Palearctic region (Gershenson and Ulenberg 1998). These moths are a useful model to decipher the process of speciation, in particular the importance of ecological adaptation driven by host-plant shifts and the utilization of species-specific pheromone mating-signals as prezygotic reproductive isolating mechanisms.

Historically the small ermine moths have presented great challenges to taxonomists due to the difficulty of identifying to species the adults of many of the *Yponomeuta*. The earliest identifications were based on larval food plants and the morphology of larvae and pupae, but this caused problems beginning with Linnaeus when he named the first *Yponomeuta* species. *Y. padella* (Linnaeus 1758) is oligophagous and feeds on *Crataegus* spp., *Prunus spinosa, P. domestica*, and *Sorbus aucuparia* but is not associated with the bird cherry *P. padus*, from which nevertheless it derives its species name *padella*. It may have been mistaken for *Y. evonymella* (Linnaeus 1758), which in spite of its name is the species feeding on *P. padus,* and not on *Euonymus* (figure 13.1 and Table 13.1). After Linnaeus, taxonomic investigations were based on examination of adult morphological characters (e.g., wing-spot size and color, genitalia) (Martouret 1966), which did not allow conclusive discrimination of all species, leading to recognition of the so-called *padellus*-species complex (Friese 1960) which later proved to include five species (Wiegand 1962; Herrebout et al. 1975; Povel 1984).

In the 1970s, "the small ermine moth project" was initiated to include research on many aspects of the small ermine moth biology. A major aim of the project was to uncover whether *Y. malinellus*, the apple orchard ermine moth, which was causing considerable damage in apple orchards in the Netherlands, was identical to ermine moths found on shrubs and other plants in the area, a question of considerable practical consequence for pest monitoring (Herrebout et al. 1975). Coordinated by Wim Herrebout and Jacobus Wiebes, at Leiden University in the Netherlands (van Bruggen and van Achterberg 2000), the research was carried out by several independent groups over two decades, including researchers from Groningen, Amsterdam, and Wageningen in the Netherlands and the University of Lund in Sweden. It resulted in 12 PhD theses and many more associated research publications (reviewed in Menken et al. 1992). The multidisciplinary

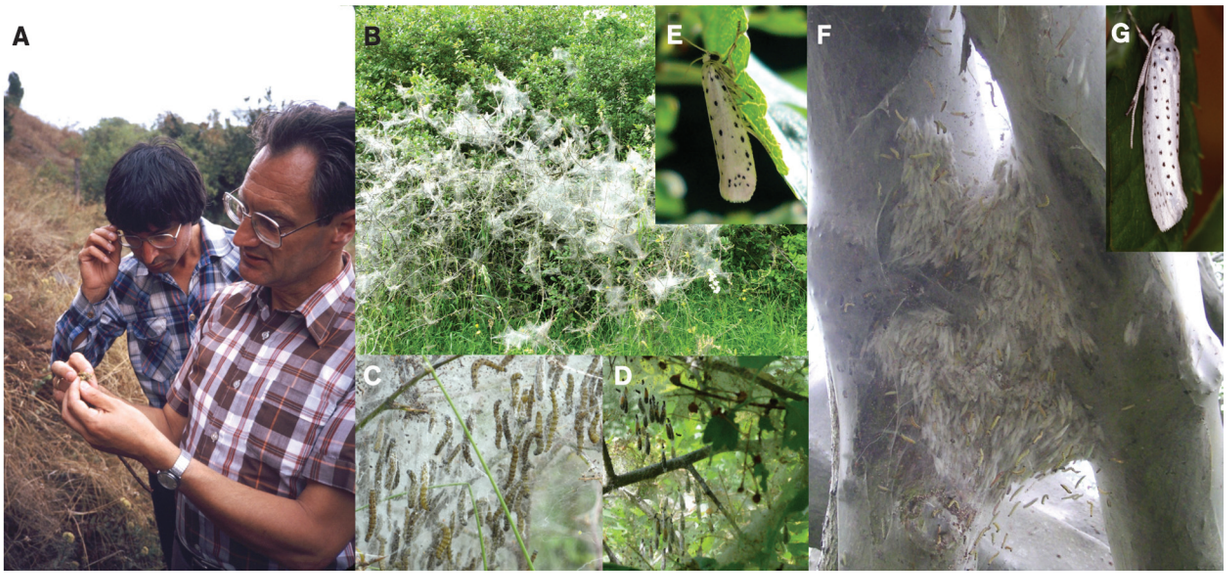

FIGURE 13.1 The small ermine moth project and some European species of small ermine moths.

A Drs. Wim Herrebout (coordinator) and Jan van der Pers (electrophysiologist and founder of Syntech), two of the scientists who took part in the initial "Small ermine moth project" launched in the 1970s.

B–C *Yponomeuta padella* nest and larvae on *Prunus spinosa* (Dalby, Skåne, Sweden).

D–E Pupae and adult *Yponomeuta padella* on *Crataegus* sp.

F Spectacular covering tree webs of larvae and pupae of *Yponomeuta evonymella* (Lund, Skåne, Sweden).

G Adult *Yponomeuta evonymella*.

PICTURE CREDITS: Christer Löfstedt (A), Marjorie Liénard (B–F), and Stephen Menken (G).

TABLE 13.1

Ecological and temporal dimensions of reproductive isolation in small ermine moths

	Ecological		Temporal
Species	Host plant (family)	Common name	Female calling period
Yponomeuta cagnagella	*Euonymus europaeus (Celastraceae)*	European spindle tree	Dawn
Yponomeuta irrorella	*Euonymus europaeus (Celastraceae)*	European spindle tree	Dawn
Yponomeuta plumbella	*Euonymus europaeus (Celastraceae)*	European spindle tree	Night
Yponomeuta rorrella	*Salix* species (Salicaceae)	Willow tree	Dawn
Yponomeuta gigas	*Salix canariensis*,§ *Populus alba* (Salicaceae)	Willow tree, silver poplar	Not known
Yponomeuta evonymella	*Prunus padus* (Rosaceae)	Bird cherry tree	Dawn
Yponomeuta mahalebella	*Prunus mahaleb* (Rosaceae)	Mahaleb cherry tree	Dawn
Yponomeuta malinellus	*Malus* sp. (Rosaceae)	Apple tree	Dawn
*Yponomeuta sedella***	*Sedum telephium* (Crassulaceae)	Stonecrops plants	Night
Yponomeuta padella	*Crataegus sp., Prunus spinosa*§ *Prunus domestica, Sorbus aucuparia* (Rosaceae)	Hawthorn, blackthorn, plum tree, rowan	Dawn

SOURCE: Adapted following Friese (1960); Wiegand (1962); Gerrits-Heybroek et al. (1978); Löfstedt et al. (1991).
** *Previously Y. vigintipunctatus.*
§ Primary host.

approach facilitated significant advances in our understanding of phylogenetic relationships of the nine European *Yponomeuta* species, their sex pheromones and host plants, and ultimately the evolution of the genus and factors driving its diversification.

Early efforts were directed to provide rigorous phylogenies by using allozyme techniques (Menken 1980). Subsequently, nine European species were recognized by multivariate analysis of both morphological and biological characters in larvae, pupae, and adults (Povel 1984). In addition to the phylogenetic and taxonomic work, larval food choice (Kooi 1990), larval taste receptors (van Drongelen 1980), larval parasitoids (Dijkerman 1990), larval trail marking (Roessingh 1989), the chemical composition of host plants (Fung 1989), and host race formation (Raijmann, 1996) were investigated in order to obtain insights into the evolution of host-plant relationships and the processes that have led to present-day associations. Finally, the chemical composition of sex pheromones (Löfstedt and van der Pers 1985; Löfstedt et al. 1986; Löfstedt and Herrebout 1988; Löfstedt et al. 1991), odor perception (van der Pers 1978, 1981, 1982; van der Pers and den Otter 1978), and the role of pheromones and host-plant volatiles in reproductive isolation (Hendrikse 1990) were also investigated, providing a comprehensive framework and establishing ermine moths as an important model for study of the evolution of sex pheromones and their role in mate recognition and speciation.

In this chapter, we summarize ecological factors of relevance to mate-finding and pheromone communication in the small ermine moths, and we review the sex pheromone studies that were mainly carried out in the 1980s. Some earlier screening studies have been left out. We also touch upon how application of molecular techniques that have become available during the last decades have more recently started to refine our understanding of the evolution and role of sex pheromone communication in this fascinating group of closely related moth species.

Some of the outstanding questions are the relative importance of host plants versus pheromones and other ecological factors in promoting the early stages of population divergence and reproductive isolation and whether the predominant factors differ between species with different ecological niches. It also remains to be determined whether present-day host-plant associations that evolved from a common ancestor associated with Celastraceae (*Euonymus*) occurred through speciation in allopatry or in sympatry through selection for pheromone divergence and host-plant shifts (e.g., driven by host race enemy-free space mechanisms or competition for resources) (Menken and Roessingh 1998).

The Evolution towards Specialized Host-Plant Associations

The genus *Yponomeuta* radiated early through sequential adaptation, i.e., following the evolution of host plants (Jermy 1984). Currently, there are 76 known species of *Yponomeuta* found almost all around the world, including Australia, New Zealand, Eurasia, Africa, Asia, and North America, except for arctic and desert regions (South America and Antarctica were not investigated) (Gershenson and Ulenberg 1998; Turner et al. 2010). The nine species found in Western Europe (see Table 13.1) occur sympatrically mainly in Palaearctic regions. A closely related 10th species, *Y. gigas*, is endemic to the Canary Islands and is not considered to be Western European and following biogeographic regions was placed among African species (Cox 2001).

Ermine moth species have strong host associations and are typically monophagous on one host or, if oligophagous, restricted to plants of the same family (Table 13.1). Consequently, host-plant selection has long been hypothesized to be an important element in the speciation process through sympatric divergence via host-race formation (Wiebes 1976; Gerrits-Heybroek et al. 1978).

Phylogenetic analyses of the genus (Menken 1996; Ulenberg 2009; Turner et al. 2010) have increased our understanding of the evolution of ermine moth host-plant associations. The Western European species are hypothesized to have evolved from a common ancestor on Celastraceae, most likely associated with the European spindle tree (*Euonymus europaeus*), a host plant which remains today in association with *Y. cagnagella, Y. irrorella*, and *Y. plumbella* (Menken et al. 1992; Turner et al. 2010). A sister taxon of the Yponomeutinae, the Saridoscelinae, also feed exclusively on Celastraceae, which supports the view that the association evolved very early in the Yponomeutidae and in the case of *Yponomeuta* spp. represents the ancestral character state (Gerrits-Heybroek et al. 1978; Ulenberg 2009). To date, host-plant family associations are known for 39 species of Yponomeutidae, among which 32 species are monophagous or oligophagous on one plant genus. At least 27 of these (more than one-third of all species in the genus) still feed on Celastraceae plants, of which 22 species are still associated with the ancestral host genus, *Euonymus* (Ulenberg 2009).

The colonization of new hosts from the Rosaceae, Crassulaceae, and Salicaceae families took place via sequential evolution (reviewed in Menken et al. 1992; Menken 1996). Mapping host plants onto the phylogeny of European *Yponomeuta* species indicates that the genus most likely dispersed from East Asia to the western Palearctic (figure 13.2). Concomitantly a single shift occurred from the ancestral Celastraceae to Rosaceae, involving an ancestor of *Y. mahalebella* and subsequently formed the clade leading to *Y. evonymella*, *Y. padella*, and *Y. malinellus* (Menken 1996; Turner et al. 2010). In contrast, Y. *plumbella*, *Y. irrorella*, and *Y. cagnagella* remained associated with Celastraceae although in the case of *Y. cagnagella*, it is very likely a secondary association following a reversal from Rosaceae (Ulenberg 2009; Turner et al. 2010) as evidenced by its relict sensitivity to benzaldehyde, a chemical abundant in Rosaceae plants but absent from its host *E. europaeus* (Roessingh et al. 2007). Finally, a shift from Rosaceae to Salicaceae is observed for *Y. rorrella* and its close relative *Y. gigas*, whereas *Y. sedella* further specialized to *Sedum* sp. (Crassulaceae) (figure 13.2) (Menken et al. 1992; Turner et al. 2010). *Y. padella* is the sole species exhibiting an oligophagous feeding pattern including several genera of Rosaceae (Table 13.1), while all other European species remain strictly monophagous.

The exact nature of the changes leading to adaptation to new hosts is unknown but could have involved enemy-free space mechanisms, the evolution of new adult host preference, and adaptations of larval host acceptance behavior including new feeding preferences, changes at the sensory level, and changes in digestive or detoxification systems (Kooi 1990; Roessingh et al. 2000). Larval gustatory sensitivity towards various host-plant chemicals was initially studied in the European species by electrophysiological recordings of larval chemoreceptor sensilla (van Drongelen 1979, 1980).

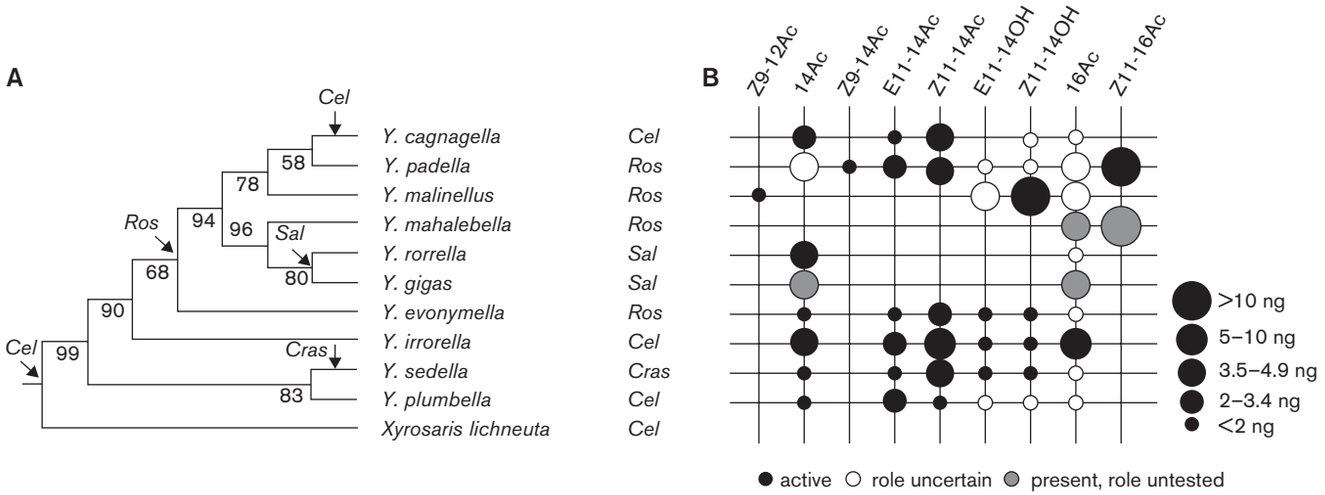

FIGURE 13.2 Phylogeny and pheromone composition.

A Phylogeny of the western European clade of small ermine moths. The MP tree was reconstructed using consensus based on the COXII, 16S, and ITS phylogenies after Turner et al. (2010), and *Xyrosaris lichneuta* as an outgroup. Host plant shifts are indicated by arrows and current affiliations are indicated after the species names. Cel, Celastraceae, ancestral host; Ros, Rosaceae; Sal, Salicaceae; Cras, Crassulaceae. Please note that *Yponomeuta sedella* was sometimes previously named *Y. vigintipunctatus*.

B Pheromone composition and titres per individual small ermine moth female, adapted from Löfstedt et al. (1991) and Löfstedt and Herrebout (1988). Filled circles represent female pheromone components with confirmed behavioral activity in conspecific males, empty circles are compounds present in the gland for which the role in mate attraction is uncertain or not essential. The sex-pheromone of *Yponomeuta gigas*, a species endemic to the Canary Islands, is similar to *Y. rorrella*. Only saturated acetates were found in pheromone gland extracts (C. Löfstedt, unpublished).

Celastraceae-feeders, i.e., *Y plumbella*, *Y. irrorrella*, and *Y. cagnagella* are sensitive to dulcitol, a feeding stimulant sugar alcohol (van Drongelen 1979; Peterson et al. 1990), whereas Rosaceae-feeders such as *Y. padella* and *Y. evonymella* respond to various degrees to sorbitol, a stereoisomer of dulcitol present at high levels in this plant family (van Drongelen 1979; Roessingh et al. 1999), and to the Rosaceae-specific compound benzaldehyde (Kooi 1988; Roessingh et al. 2007). Although adult females mainly determine the host plant by their choice of oviposition sites in *Yponomeuta*, it has been proposed that the shift from Celastraceae to Rosaceae could also have been facilitated by larval preferences toward the presence of low amounts of dulcitol in Prunoidea, a suborder of Rosaceae (Menken and Roessingh, 1998). Since Prunoidea also contain sorbitol, and both dulcitol and sorbitol are perceived in homologous peripheral sensory cells, a simple sensory shift might have favored the acquisition of a new feeding preference allowing the utilization of Rosaceae as new host plants (Menken and Roessingh, 1998; Roessingh et al. 1999). Interestingly, *Y. mahalabella*, *Y. padella*, and *Y. evonymella* are still sensitive to dulcitol (van Drongelen 1979) despite feeding on Rosaceae hosts containing no or very limited amounts of this sugar, which supports the idea that ancestral larval preferences are maintained among *Yponomeuta* (van Drongelen 1979). As mentioned above, although feeding on Celastraceae, sensory cells in larval maxillary palps of *Y. cagnagella* respond strongly to benzaldehyde, supporting a recent reversal event from a Rosaceae host-plant ancestor common with *Y. padella* (Roessingh et al. 2007; Turner et al. 2010).

To corroborate the current correlations between the phylogenetic pattern and the role of sensory stimuli adaptation in ecological speciation, future studies should further investigate adult oviposition preferences and larval sensory responses to host specific plant compounds.

Sex Pheromones and Other Ecological Factors Involved in Reproductive Isolation

Overview of Sex-Pheromone Composition

Female sex pheromones were demonstrated in the early 1980s to play an essential role in efficient mate finding in small ermine moths (reviewed in Löfstedt et al. 1991). Females bend their abdomen in a characteristic position that leads to the extrusion of the last abdominal segments (figure 13.3A) (Hendrikse 1978), actively displaying the pheromone gland and thereby releasing the pheromone into the surrounding air.

Like many other closely related moth species, *Yponomeuta* sex pheromones are blends of structurally related C_{14} and C_{16} acetates and alcohols (figure 13.2) (Löfstedt et al. 1991). The (*Z*)-11-tetradecenyl acetate (Z11-14Ac) likely represents an ancestral pheromone component based on its occurrence across the *Yponomeuta* genus (Löfstedt and van der Pers 1985; Löfstedt and Herrebout 1988; Löfstedt et al. 1991). Six of the nine European species, including the four basal species in the clade (*Y. plumbella*, *Y. sedella*, *Y. evonymella*, and *Y. irrorella)* as well as *Y. padella* and *Y. cagnagella* use a mixture of Z11-14Ac together with varying ratios of (*E*)-11-tetradecenyl acetate (E11-14Ac) (figures 13.2 and 13.4) as primary pheromone components, i.e., pheromone components that cannot be subtracted from the synthetic pheromone without resulting in a significant loss of activity. Additional gland constituents (and likely pheromone components) include combinations of tetradecyl acetate (14Ac), (*Z*)-9-tetradecenyl acetate (Z9-14Ac), and (Z)-11-hexadecenyl acetate (Z11-16Ac) as well as the corresponding unsaturated C_{14} alcohol compounds, with or without confirmed behavioral activity depending on the species (Löfstedt et al. 1991). Some of the compounds display a more repeatable average ratio than others, which may indi-

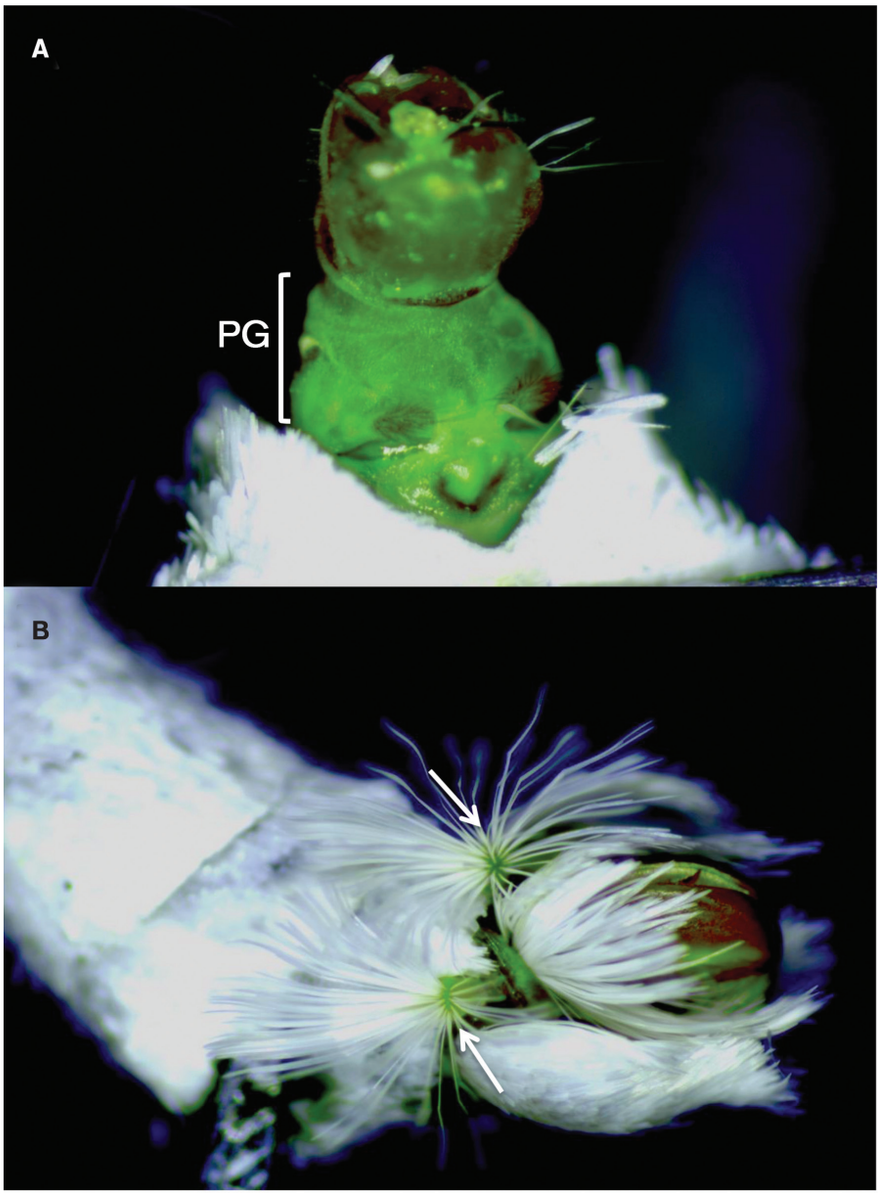

FIGURE 13.3 *Yponomeuta rorrella* female pheromone gland and male hairpencil structures.

A Ventral view of an *Yponomeuta rorrella* female extruding her 8th and 9th to 10th terminal abdominal segments; the sex pheromone gland (PG) is located along the intersegmental integument between the 8th and 9th abdominal segments.

B Lateral view of an *Yponomeuta rorrella* male with extruded hairpencil-like structures. Whether these structures are vestigial or play a role in courtship behavior, as suggested by Hendrikse et al. (1984), remains to be confirmed.

PICTURE CREDITS: Jean-Marc Lassance.

cate evolutionary constraints due to their importance for reproductive isolation (Löfstedt and van der Pers 1985; Du et al. 1987; Löfstedt and Herrebout 1988) or simply reflect the nature of the biosynthetic pathways involved in their production. For instance, low variation was found in the production of E11/Z11-14Ac components in *Y. padella*, in contrast to higher variation found among saturated acetates and the unsaturated (*Z*)-9-tetradecenyl acetate (Z9-14Ac) in repetitive sampling of individual females (Du et al. 1987).

The species *Y. cagnagella, Y. irrorella*, and *Y. plumbella* share *Euonymus* as a host plant and like all European *Yponomeuta* they are sympatric and in this case also synchronic. The pheromones of these three species are blends of several compounds in addition to Z and E11-14Ac. The pheromone blend of *Y. plumbella* also contains saturated acetates. Female *Y. plumbella* and *Y. irrorella* emit a mixture of 16Ac, 14Ac, E-, and Z11-14Ac. The former emit a 25:46:148:100 ratio, whereas the latter emit a 17:68:56:100 ratio (figure 13.2). The *Y. cagnagella* pheromone blend consists of three components, 14Ac, E11-14Ac, and Z11-14Ac, in a 30:3:100 ratio (Löfstedt and Herrebout 1988). The saturated tetradecyl acetate acts as a synergist in all three species, whereas 16Ac is produced in all species

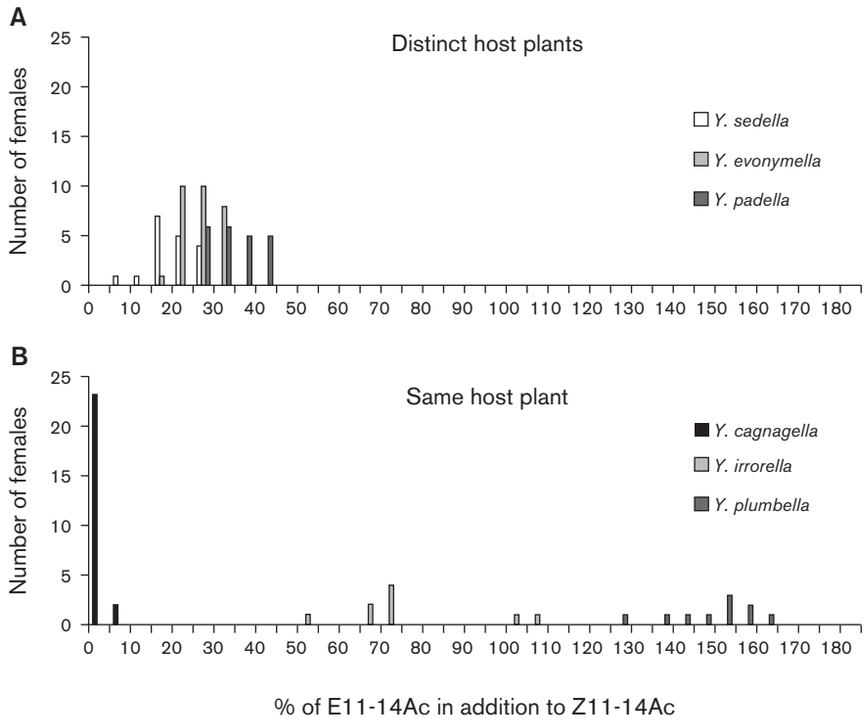

FIGURE 13.4 Frequency distribution of pheromone production in individual females in relation to cross-attraction and host plant. The graphs represent the E11- to Z11-14Ac isomer ratios in individual female pheromone gland extracts analyzed by gas chromatography, from six different Yponomeuta species.

A In species that do not share a host plant, cross-attraction has been observed in flight tunnel experiments as a consequence of overlapping relative amounts of E11-14Ac.

B The three species sharing the spindle tree as host plant show a pattern of reinforcement through adaptive selection for pheromone differences; non-overlapping ranges of E11- to Z11-14Ac actively prevent cross-attraction in flight tunnel experiments and in the field.

SOURCE: Adapted from Löfstedt et al. (1991).

but has confirmed behavioral activity only in *Y. irrorella*. Female glands in all three spindle tree species also contain tetradecanol and the corresponding monounsaturated alcohols (figure 13.2), but the alcohols only appear to be active minor components in *Y. irrorella* (Löfstedt and Herrebout 1988). The range of Z/E11-14Ac ratios produced by each species is essential for conspecific male attraction by females of the three species sharing the spindle tree as host plant. Moreover, subtraction of E11-14Ac in either of the synthetic pheromones of *Y. irrorella* or *Y. plumbella* leads to high catches of *Y. cagnagella* in field-trapping experiments (Löfstedt and Herrebout 1988). The likely explanation is that a majority of *Y. cagnagella* females produce a very low amount of E11-14Ac that averages 3% relative to the Z11-14Ac. Thus, even a trace amount of E11-14Ac occurring as an impurity in the synthetic Z11-14Ac may still be enough to cause significant attraction of *Y. cagnagella*.

Whereas the *Y. cagnagella* and *Y. plumbella* pheromones are highly specific due to very distinct E/Z isomer ratios, the *Y. irrorella* synthetic blends consistently attracted low numbers of *Y. cagnagella* and *Y. padella* in the field, demonstrating that mate discrimination and reproductive isolation are not entirely mediated by pheromones but require additional isolating mechanisms (i.e., different hosts for *irrorella* and *padella*, possibly different time and height of flight [Herrebout and van de Water 1983]) and possibly male courtship pheromones, although the latter has not been conclusively demonstrated (Löfstedt and Herrebout 1988).

The two allochronic species, *Y. evonymella* and *Y. sedella*, are nearly identical with respect to their female-produced pheromones. They produce a 100:20 mixture of Z11-14Ac and E11-14Ac, and in addition minor amounts of Z/E11-14OH are reported to synergize trap catches (Löfstedt and van der Pers 1985). Although the E11-14Ac/Z11-14Ac ratio of individual *Y. padella* females overlaps with those of *Y. evonymella* and *Y. sedella* (figure 13.4), *Y. padella* females produce a 34:100:400 mixture of E11-14Ac, Z11-14Ac, and Z11-16Ac (Löfstedt and van der Pers 1985) and the presence of Z11-16Ac efficiently reduces trap catches of *Y. evonymella* and *Y. sedella* (Löfstedt and van der Pers 1985). *Y. padella* is also unique in having small amounts of Z9-14Ac as an important fourth active component (Löfstedt et al. 1991).

Among the three remaining species, *Y. malinellus* uses an unusual combination of (*Z*)-9-dodecenyl acetate (Z9-12Ac) and Z11-14OH (McDonough et al. 1990), whereas *Y. mahalebella* (only female glands analyzed, no behavioral experiments) and *Y. rorrella* differ by an overall reduced number of acetates and a decrease in pheromone complexity that likely played a role in the adaptation to new communication niches (Löfstedt et al. 1991). *Y. mahalebella* females produce Z11-16Ac plus saturated acetates, whereas *Y. rorrella* females produce a mixture of saturated acetates, of which only 14Ac has been

demonstrated to be attractive to males. Among all European small ermine moth species, the *Y. rorrella* pheromone is unique due to the absence of any unsaturated compounds (figure 13.2) and any unsaturated fatty acyl precursors (Löfstedt et al. 1991), pointing to a mechanism disrupting Δ11-desaturation in this species (Löfstedt et al. 1986).

Temporal and Behavioral Niches Contributing to Species Separation

Interspecific encounters are common among European small ermine moths in their natural habitats. Cross-attraction occurs between several species under field (see above) and laboratory conditions (figures 13.4 and 13.5). This reflects similarities in female pheromone production (figure 13.2), mirrored by *Yponomeuta evonymella* males showing strong responses to synthetic blends mimicking *Y. padella*, *Y. irrorella*, and *Y. sedella* female pheromones in the wind tunnel, and males of *Y. padella* responding to *Y. irrorella* synthetic pheromones in the field (figure 13.5) (Löfstedt and Herrebout 1988; Löfstedt et al. 1991). However, hybridization under natural conditions has rarely been reported; among trapping studies that captured tens of thousands of males, discriminant allozyme analyses never identified a single hybrid (Hendrikse 1979). Depending on species, additional prezygotic (e.g., flight height, temporal differences in diurnal and seasonal activity, male courtship behavior) and postzygotic isolating mechanisms may contribute additional dimensions to the pre-mating reproductive isolation provided by sex pheromones.

As mentioned earlier, *Y. evonymella* and *Y. sedella* have a more or less identical pheromone composition (figure 13.2) (Löfstedt et al. 1991), likely accountable for by the absence of selection for pheromone divergence due to differences in geographic distribution and seasonal activity. *Y. evonymella* is active at dawn, whereas *Y. sedella* is active in the middle of the night (Hendrikse 1979). *Y. evonymella* is also univoltine, flying from late June to mid-July in south Sweden, at approximately the same time as most of the other small ermine moths, whereas *Y. sedella* is bivoltine with its first flight occurring well before the flight of *Y. evonymella,* and the second one late in the summer (Löfstedt and van der Pers 1985; Löfstedt et al. 1986; Löfstedt and Herrebout 1988).

Temporal differences can additionally reinforce niche separation. For instance, *Y. plumbella* and *Y. sedella* females call early in the night compared to females of all other species, which preferentially call near the end of the scotophase and at dawn (Hendrikse 1979). Female maturation (i.e., peak in calling activity) varies depending on species from 1 day (*Y. plumbella*) to 10 days (*Y. malinellus* and *Y. cagnagella*) and averaging 2–5 days after emergence for other species (Hendrikse 1979), which may also contribute to temporal niche distinctness.

Regarding the potential role of male pheromones, all *Yponomeuta* males possess two sets of abdominal, eversible hairpencils (see figure 13.3B) that they extrude during courtship while wing fanning at close range toward calling females (Hendrikse 1979). This behavior suggested that male-species-specific signals might help females to discriminate among mates (Hendrikse et al. 1984) and function to enhance reproductive barriers, particularly for those species for which cross-attraction has been observed. In flight tunnel experiments, Hendrikse et al. (1984) demonstrated that *Y. padella* and *Y. evonymella* females rejected more than 90% of heterospecific

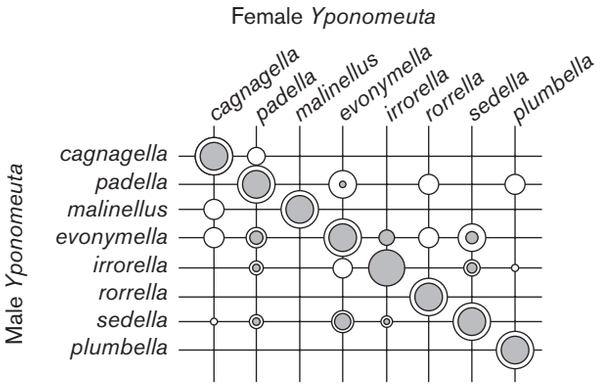

FIGURE 13.5 Male behavioral response to calling females (figure modified after Hendrikse 1986 and Löfstedt et al. 1991). Empty and full circles represent males taking flight and landing, respectively. Response is proportional to the diameter of circles (intraspecific empty circles correspond to 100%).

males. Furthermore, results with *Y. padella* suggested that the male hairpencil display might mediate female mate recognition and acceptance. An inhibitory effect on other males was also noted in *Y. cagnagella, Y. evonymella*, and *Y. padella*. When males actively displaying their hairpencils were placed upwind of a calling female, wing-fanning movements in conspecific and heterospecific males placed downwind were decreased (Hendrikse et al. 1984). Although this moth family is not known to possess ears (Scoble 1992), it was not possible to discriminate among the competing hypotheses that acoustic or olfactory cues were involved. Despite behavioral indications, a male pheromone in *Y. padella* acting as a second pre-mating isolation barrier especially against interspecific hybridization with *Y. irrorella* and *Y. evonymella* females (Löfstedt and Herrebout 1988) has not yet been conclusively demonstrated.

Most European ermine moth species have species-specific differences in their female pheromone blends. Whenever pheromones remain similar, temporal and behavioral factors play a more important role. With all ecological factors combined, all nine European species occupy a virtually unique communication channel that ensures reproductive isolation.

Pheromone Biosynthesis and Modulation of Blend Ratios

The biosynthetic machinery of mate signaling in moths is controlled by several multigene families (see Roelofs and Rooney 2003; Blomquist et al. 2005). Fatty-acyl-CoA desaturases and fatty-acyl-CoA reductases have been characterized and confirmed to encode specific enzymatic functions that, in addition to uncharacterized β-oxidases, acetyl-transferases, and oxidases, contribute to defining the final structures and ratio of each component in the species-specific pheromone mixtures (Roelofs and Bjostad 1984; Bjostad et al. 1987). Similar to most moth species for which pheromone biosynthetic pathways have been elucidated, the production of unsaturated components in the *Yponomeuta* spp. pheromone blends was postulated to start from the ubiquitous palmitic acid (hexadecanoate or 16:Acyl) and involve combinations of chain shortening and desaturation by a Δ11-desaturase (Löfstedt et al. 1991). The proposed biosynthetic pathway (figure 13.6) is based on analyses of fatty-acyl precursors in pheromone gland extracts (Löfstedt et al. 1991). In addition to

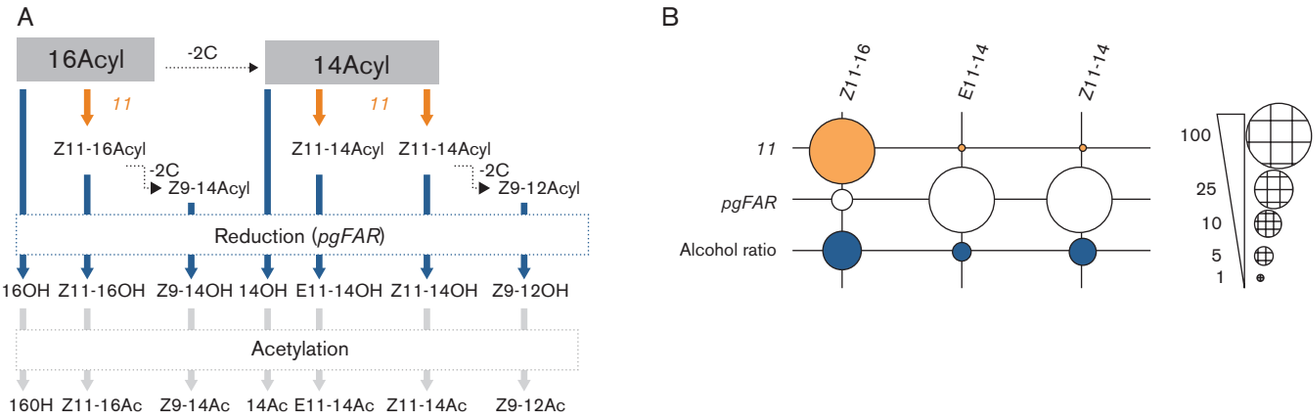

FIGURE 13.6 Pheromone biosynthesis and modulation of pheromone ratios in small ermine moths.

A Pheromone biosynthesis pathway including characterized biosynthetic genes (Δ11, delta11 acyl-CoA-desaturase; pgFAR, pheromone gland specific fatty-acyl-CoA-reductase). Pheromone production starts from palmitic acid (16Acyl) and all unsaturated acyl precursors are synthesized by combination of Δ11-desaturation (Δ11) and β-oxidation (-2C). Subsequently, a single pgFAR reduces the species-specific precursors present in the gland of the respective species to produce intermediate alcohol products as investigated in *Yponomeuta evonymella*, *Y. padella*, and *Y. rorrella* (Liénard et al. 2010) that are acetylated to give the active pheromone compounds. Note that the only European species producing Z9-12Ac as pheromone compound is Y. *malinellus*, and the pathway represented here leading to Z9-12Ac has not yet been confirmed in vivo.

B The reverse chain-length preference of the Δ11-desaturase and pgFAR for acyl substrates with 14 or 16 carbon atoms in *Yponomeuta padella* allows adjusting the alcohol blend ratio, which matches the active blend ratio especially for Δ11-unsaturated compounds. The circled areas are proportional (%) to the *Y. padella* Δ11-desaturase (upper line) and pgFAR substrate preferences (middle line), and to the final ratio between components (bottom line) (adapted from Liénard and Löfstedt 2010).

16:Acyl, precursor analyses revealed the presence of myristic acid (tetradecanoate or 14:Acyl), (*Z*)-9-hexadecenoate (Z9-16:Acyl), and (*Z*)-9-tetradecenoate (Z9-14:Acyl) in all species. (*Z*)- and (*E*)-11-tetradecenoates are found in all species but *Y. rorrella* and *Y. mahalebella*. (*Z*)-11-hexadecenoate (Z11-16:Acyl) is present in *Y. cagnagella*, *Y. padella*, and *Y. mahalebella*. Finally, (*Z*)-9-dodecanoate (Z9-12:Acyl) is found only in *Y. malinellus* (Löfstedt et al. 1991). The species-specific pools of saturated and monounsaturated fatty-acyl precursors are subsequently reduced and acetylated.

Although no experiments with labeled precursors have been carried out in vivo to support the postulated pathways, some of the biosynthetic enzymes have been isolated and functionally characterized, including a pheromone gland-specific Δ11-fatty-acyl-CoA desaturase and a broad-range fatty-acyl-CoA reductase (pgFAR) acting on C_{14} and C_{16} acyl precursors (Liénard and Löfstedt 2010; Liénard et al. 2010). The Δ11-desaturase characterized from *Y. padella* produces large amounts of (*Z*)-11-hexadecenoic acid but also catalyzes the dehydrogenation of tetradecanoic acid to produce minor amounts (around 5% of the produced unsaturated FAs) of (*E*)- and (*Z*)-11-tetradecenoic acids, altogether accounting for the production of all potential intermediate Δ11 fatty-acyl-precursors (Liénard and Löfstedt 2010). The broad range, exquisitely nonselective pgFAR has been functionally characterized in *Y. padella, Y. evonymella*, and *Y. rorrella* and accounts for the reduction of a wide range of saturated precursors (from C_{12} to C_{16}) and their corresponding Δ9 and Δ11 unsaturated acyl precursors, thus including all potential saturated and monounsaturated fatty-acyl-precursors found across the *Yponomeuta* genus (Liénard et al. 2010).

Pheromone glands of *Y. rorrella* females differ markedly from other ermine moths by their absence of any Δ11 unsaturated fatty-acyl precursors, suggesting that the Δ11-desaturase is inactive in this species (Löfstedt et al. 1986), yet the exact molecular mechanism remains unknown. Nevertheless, in agreement with the scenario under which the simple *Y. rorrella* pheromone blend derives from an ancestral more complex multicomponent pheromone (Löfstedt et al. 1986), its downstream pgFAR enzyme has retained the ability to reduce all unsaturated compounds. This also suggests that FARs might have a conserved function, if not throughout the genus at least in several of the ermine moth species (Liénard et al. 2010). In *Y. padella*, the pgFAR substrate specificity ($C_{14} > C_{16}$) further counterbalances the inherent chain-length preference ($C_{16} > C_{14}$) of the upstream Δ11-desaturase to modulate the production of the unsaturated alcohols. In vivo, the alcohol outcome depends on the species-specific fatty-acyl pools (Liénard et al. 2010), but essentially the Δ11-alcohol ratios correlated with the final acetate ratios previously reported (Löfstedt et al. 1991).

Among all nine species only female *Y. malinellus* use Z9-12Ac as a sex pheromone compound together with Z11-14OH (McDonough et al. 1990). However, female pheromone glands in that species also contain E11-14OH, 14OH, 16OH, and 16Ac but neither saturated nor unsaturated C_{14} acetates (McDonough et al. 1990). The presence of these compounds in female glands supports the hypothesis that pgFAR has broad range activity similar to that of *Y. padella*, *Y. evonymella*, and *Y. rorrella* and a selective acetyl transferase that does not acetylate the C_{14} alcohols. In addition, this species likely possesses a β-oxidase able to chain-shorten the Z11-14:Acyl to produce the Z9-12:Acyl otherwise absent in other species.

Pheromone biosynthesis in small ermine moths involves several key biosynthetic enzyme-encoding genes, including a single Δ11-desaturase and a single pgFAR. Characterization of these enzymes has shed light on some of the mechanisms involved in determination of the final blend composition in several of the species (Liénard and Löfstedt 2010). Candidate genes involved in chain shortening and acetylation remain to be identified and may reveal the mechanisms involved in adjusting the final ratios between unsaturated and saturated components, and between alcohols and acetates.

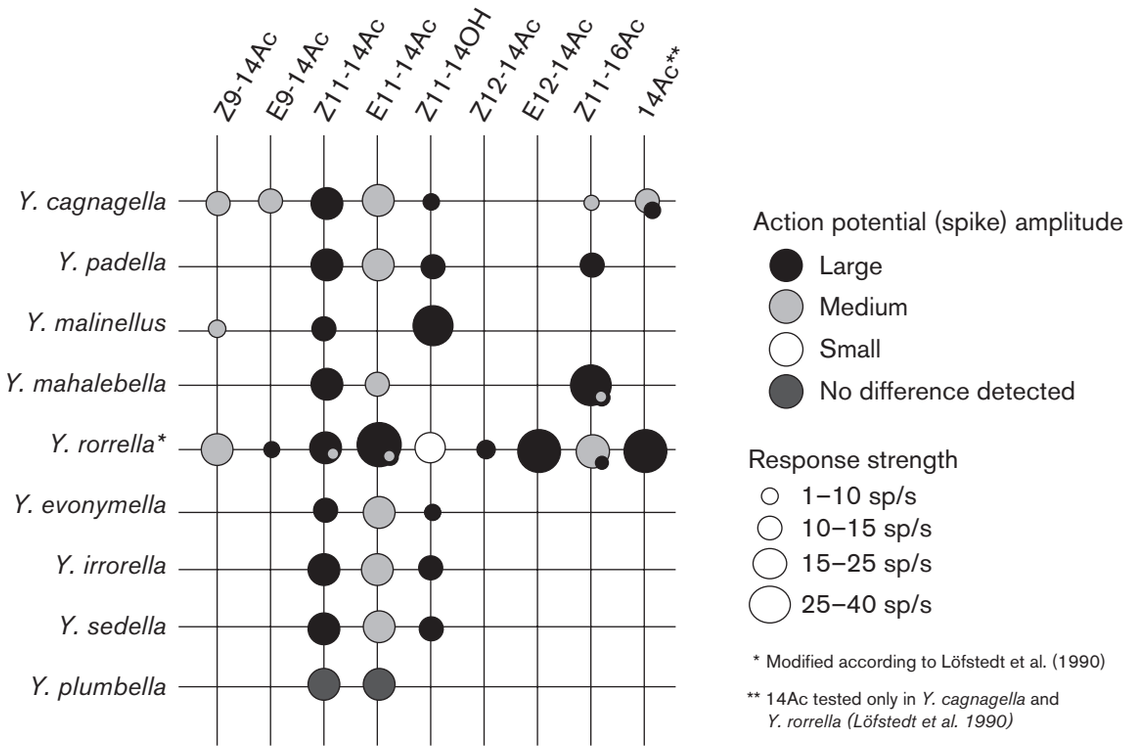

FIGURE 13.7 Electrophysiological responses of male sensilla trichoidea to pheromone compounds. Responses of olfactory sensory neurons to pheromone compounds in nine species of small ermine moths. Activities elicited in receptor cells are presented for the most abundant sensillum type (type I) that harbors one or two cells with different spike amplitudes. The size of a circle reflects the response to 1 µg (van der Pers 1982) or 10 µg (Löfstedt et al. 1990) of doses of the stimulus. The bulk of data is extracted from van der Pers (1982), but the 14Ac and *Yponomeuta rorrella* data as indicated by asterisks (except E9-14Ac and Z11-14OH) are modifications based on Löfstedt et al. (1990).

Male Physiological and Behavioral Response

Detection of Pheromone and Plant Compounds

Like many other moth species, male small ermine moths are capable of detecting host-plant odors and conspecific pheromone components, the latter through specialized pheromone receptor cells localized in sensilla trichodea (Cuperus 1985). Interactions between pheromone compounds and plant odors were studied by electroantennogram (EAG) and single cell recordings, demonstrating that responses of pheromone compound receptors in males were stronger in the presence of host-plant odors (van der Pers et al. 1980). This suggested that a synergism between the two types of odors might occur in the field, although this has not been demonstrated. The observation is nevertheless interesting because of the possible interplay between pheromones and host plants in the process of speciation.

Antennal response profiling was carried out using EAG and single sensillum tip-recording techniques. The results were based on a variable number (8–36) of randomly selected sensilla per species. The first pheromone identifications had not been reported yet and each recorded sensillum was assessed for a broad range of potential pheromone compounds including monounsaturated and doubly unsaturated C_{14}, C_{15}, and C_{16} acetates as well as Z11-14OH (van der Pers 1982). The doubly unsaturated compounds and the C_{15} acetates were never found in female pheromone gland extracts (Löfstedt et al. 1991), and thus responses to these compounds will not be discussed further. The importance of saturated acetates as pheromone components in some of the species was not known at the time and these are not included in the study by van der Pers (1982) but were tested later in *Yponomeuta rorrella* (see below) (Löfstedt et al. 1990).

In European ermine moth species, male antennae consist of approximately 60 segments that harbor curved sensilla trichodea ranging from 20 to 30 µm in length, and in which are housed olfactory sensory cells. In seven of the nine species, two types of sensilla with different response profiles were categorized as type I and type II. Type I sensilla are the most abundant type and their physiological response spectra are compiled in figure 13.7 (van der Pers and den Otter 1978; van der Pers 1982; Löfstedt et al. 1990). *Y. cagnagella* and *Y. irrorella* possess a third type of sensillum (type III), which has a characteristic response profile distinct from types I and II (van der Pers 1982). Typically, within each sensillum, two cells—olfactory sensory neurons (OSNs) firing with different action potential amplitudes named as Large (L), Medium (M), or Small (S) amplitude (spike) cells and with different response profiles—are activated upon odor stimulation (van der Pers 1978, 1982). Cells with the second largest spike amplitude are always called medium. Thus small spike amplitude cells are only recognized in sensilla with three cells. Recordings from *Y. plumbella* OSNs did not show spike amplitude differences, and thus it could not be determined how many OSNs were housed per sensillum in this species (van der Pers 1982). Another exception was found in *Y. rorrella* where type I sensilla consistently exhibited an additional small spike amplitude activity, suggesting the presence of a third OSN, preferentially responding to Z11-14OH. Altogether, the presence of

2–3 types of sensilla that each normally house 2 OSNs suggests that small male ermine moths typically possess at least 6 distinct types of OSNs.

The response profile of OSNs from the most abundant type of sensillum (type I) varies between species, but each OSN is usually sensitive to several stimuli (figure 13.7).

All sensillum types were found to respond to E11-14Ac and Z11-14Ac in all species but *Y. malinellus*, where no OSN responds to E11-14Ac. This is not surprising since female *Y. malinellus* produce a unique blend composed of Z11-14OH and Z9-12Ac, but Z9-12Ac was not included in the set of stimuli tested by van der Pers (1982). However, among all species tested, the strongest response to Z11-14:OH in OSN-L, in sensilla types I and II, were recorded in *Y. malinellus*.

In all species but *Y. rorrella*, responses to E11-14Ac are exclusively elicited via medium spike amplitude neurons in both types of sensilla, whereas responses to Z11-14Ac are exclusively elicited in large spike amplitude sensory neurons, indicating that a specific sensory neuron responds to each of the two most important pheromone components found across the genus.

OSNs responding to Z11-16Ac were found in *Y. cagnagella, Y. padella, Y. rorrella*, and *Y. mahalebella* (van der Pers 1982). Z9-14Ac elicited various degrees of responses in the sensillum type I in *Y. cagnagella, Y. malinellus, and Y. rorrella* (figure 13.7) and in a less abundant type of sensillum (type II, not shown in figure 13.7) in *Y. evonymella* and *Y. sedella* (van der Pers 1982). Intriguingly, no Z9-14Ac-tuned OSN was found in *Y. padella*, although Z9-14Ac was later confirmed to be a fourth pheromone component in that species, suggesting that OSNs responding to this compound exist that were not identified in earlier studies.

In *Y. rorrella*, the three types of sensory neurons were investigated in detail (Löfstedt et al. 1990). In this species the medium spike amplitude neurons in types I and II sensilla respond to Z9-14Ac and Z11-16Ac. In addition to responding to the pheromone component 14Ac, the large spike neurons respond to E6-, E9-, E11-, E12, Z6-, Z11-, and Z12-14Ac to varying extents. E6-, E12, Z6-, and Z12-14Ac are not found as pheromone components in any *Yponomeuta* species (Löfstedt et al. 1990) and this response profile is unusually broad compared to antennal responses in other small ermine moths. These results showing that a single OSN with large spike amplitude concomitantly responds to 14Ac, Z11-14Ac, and E11-14Ac differ slightly from the original study by van Der Pers (1982), reporting that like other ermine moths, Z11-14Ac and E11-14Ac were activated by distinct cells with large and medium spike amplitudes (figure 13.7). More generally, response profiles also vary within species, which can reflect functional intraspecific variation; differences in OSN action potential amplitudes may also differ between studies and individuals depending on the sensillum actually recorded from and the technique used. Differences in recorded action potentials from the tested sensillum can first be accounted for by the morphological properties of the sensillum itself. In the European corn borer, receptor cells with larger diameter have been shown to produce spikes with greater amplitudes (Hansson et al. 1994), a correlation that may raise the neuron sensitivity by allowing more receptor sites to coexist on the cell surface and has been proposed as an explanation as to why larger spike neurons also show higher spike frequencies (Cossé et al. 1995). Additionally, recordings of OSN activity in ermine moths were done by the cut sensillum tip recording technique, a technique which in contrast to tungsten electrode recordings may not have reached close enough to the source of the action potential (Olsson et al. 2010), possibly adding to variation in measurements of spike amplitudes between studies. Although we have gained a good general understanding of olfactory response profiles in *Yponomeuta*, some unknowns remain (i.e., Z9-14Ac response profiles are likely incomplete), together with subtle variations between datasets that may be both biological and technical. A comprehensive study reexamining the olfactory response profiles of *Yponomeuta* using the most recent techniques is needed.

Role of Antagonists as Enhancers of Reproductive Isolation and Interspecific Interactions

The presence of unsaturated acetates (Z11-14Ac and E11-14Ac or Z11-16Ac) is essential for male attraction in all small European ermine moth species but one (Löfstedt et al. 1986, 1991). *Yponomeuta rorrella* is the only *Yponomeuta* species—as a matter of fact the only moth—in which the primary pheromone component is a saturated acetate (14Ac). In this species, unsaturated compounds act as antagonists and the addition of as little as 1% of Z11-14Ac in synthetic pheromone blends dramatically lowers trap catches in the field (Löfstedt et al. 1986). This is a striking example of a pheromone component from one species being a strong antagonist for a closely related species. Although unsaturated 14-carbon acetates and alcohols have not be demonstrated to be part of the active blends in *Y. rorrella* and *Y. mahalebella,* males in both species possess receptor neurons responding strongly to these compounds and eliciting antagonist effects on behavior (van der Pers 1982; Löfstedt et al. 1990). In *Y. rorrella*, the Z11-14OH also acts as an antagonist and activates a specific OSN exhibiting a small spike amplitude (Löfstedt et al. 1986, 1990). Likewise the Z11-16Ac, an essential component for the attraction of *Y. padella* and the major pheromone compound in extracts of *Y. mahalebella,* acted as an antagonist for other sympatric ermine moth species in field experiments with synthetic compounds (Löfstedt and van der Pers 1985).

Male moths have a remarkable ability to distinguish between pheromone sources even when placed extremely close together (Valeur and Löfstedt 1996, and references therein). Despite this, pheromone sources have been observed to interfere with each other under field conditions (see for instance Perry and Wall 1984). In traps baited with female moths, addition of a *Y. malinellus* female to a *Y. padella* female suppressed the attraction of conspecifics in the same way as a *Y. padella* female suppressed the attraction of *Y. cagnagella* males to conspecific females (Minks et al. 1977). Intraspecific and interspecific interactions were further studied in a laboratory flight tunnel. By creating overlapping plumes of pheromones from different species, it was found that *Y. padella* had a more severe effect on *Y. cagnagella* than vice versa and that *Y. sedella* was not as strongly influenced by *Y. padella* as *Y. cagnagella*. *Y. cagnagella* appeared to be influenced by Z11-16Ac in the *Y. padella* pheromone but even more so by the "wrong" ratio of E11-/Z11-14Ac (Löfstedt 1987). Although these experiments, both the ones in the field and in the laboratory, simulated a rather artificial situation, populations of small ermine moths may locally be very high and individual females may call in close proximity to each other. Thus, there likely exists a real challenge for male small ermine moths in handling more or less overlapping plumes in the olfactory landscape.

Subtraction of Z11-14Ac or E11-14Ac fully abolishes intraspecific attractiveness of synthetic pheromone blends for six of the

species and subtraction of E11-14Ac or off Z/E ratios increases heterospecific trap catches (Löfstedt and Herrebout 1988). Interestingly, subtraction of any of the five components of the synthetic pheromones of *Y. evonymella* and *Y. sedella* not only reduces their attractiveness but also causes significant attraction of several tortricid moth species. For instance, subtraction of Z11-14OH or Z11-14Ac from the *Y. sedella* pheromone attracts *Aphelia paleana* or *Dichrorampha petiverella,* respectively, whereas subtraction of E11-14Ac from the *Y. evonymella* pheromone elicits attraction of *Tortrix viridana* (Löfstedt et al. 1991). Similarly, when Z11-14Ac is absent from the *Y. irrorella* pheromone blend, it attracts *Ethmia punctella* (Ethmiidae) and *Croesia holmiana*, another tortricid (Löfstedt and Herrebout 1988).

Summary: An Emerging Model System in Research on the Role of Sex Pheromones in Speciation—Toward a New "Small Ermine Moth Project"?

The small ermine moth genus *Yponomeuta* has a worldwide distribution and includes nine species that coexist sympatrically across Europe. European species are to a large extent sympatric but have evolved unique combinations of host plants and/or daily or seasonal activity patterns and/or species-specific female sex pheromones. Cumulatively, these differences ensure efficient pre-mating isolation in their natural habitat and make the European *Yponomeuta* species complex a model system to study the role of insect–plant relationships and pheromones in reproductive isolation and speciation in moths.

However, the model system has turned out more complex to study than originally thought. Therefore, the relative importance of host plants versus pheromones and possibly other factors in population divergence, speciation, and reproductive isolation has remained difficult to determine. We conclude by summarizing some of the difficulties encountered and suggest some areas of study that would enhance our understanding of the role of plants and pheromones in this system.

Overcoming the System Limitations

First, a major limitation has been the difficulty of establishing the small ermine moths in laboratory cultures due to an obligate diapause and the need to rear larvae on their host plant. Consequently, basic studies are still needed on the pheromone composition, physiology, and behavior for some of the species including *Yponomeuta mahalebella* and *Y. gigas*, a sister species endemic to Canary Islands, about which very little is known. In the case of *Y. malinellus*, the population studied by McDonough et al. (1990) was introduced to North America, and European populations should be studied as well.

Second, we do not know whether speciation in *Yponomeuta* is any more "ongoing" than in any other taxon. Small ermine moth species originally used Celastraceae as host plants and the genus expanded throughout Europe through successive host-plant shifts to Rosaceae, Crassulaceae, and Salicaceae. The evolution of female host-plant choice and larval host acceptance were likely important drivers, yet may have involved distinct sensory and behavioral changes (e.g., Menken and Roessingh 1998). Current evidence does not support the hypothesis that these adaptations alone have been decisive in driving reproductive isolation. Among all European species, *Y. padella* is the only oligophagous taxon (*Prunus spinosa*, *Crataegus* spp., *Sorbus aucuparia*, *Prunus domestica*), and we still know little of whether this oligophagous feeding pattern can be explained by larval adaptation or if the species represents a mosaic of genetically divergent populations associated with a single host. Studying model communication systems that are polymorphic and potentially undergoing current evolutionary changes may uncover the mechanisms of reproductive isolation in populations that are used as models of an early stage of speciation (Via 2009). In the case of the European small ermine moths, we still lack dating of the nodes in the phylogenetic tree, and as a result lack a time frame for the process of speciation: contemporary species in this genus might already have been formed a million years ago or, alternatively, we may face a process of "ongoing speciation." The discovery of polymorphic pheromone populations in the process of divergence or host race formation (Menken 1981) would facilitate conclusive studies.

Third, sex pheromones most likely played a significant role in the evolution of reproductive isolation in *Yponomeuta* since, except for two allochronic species, all taxa share habitats—or host plants—and overlap in periods of sexual activity. Species-specific blends and ratios of female-produced pheromones and the occurrence of pheromone components with an antagonistic behavioral effect on males of other species evidently prevent cross-attraction and hybrid formation in the field that can otherwise occur under laboratory conditions. In support of this scenario, many features of the variation in the pheromone systems of small ermine moths can likely be assigned adaptive explanations, while others cannot. Some will require further work.

Possible Areas of Future Study

(A) Evolution towards simpler pheromones with *Yponomeuta rorrella* as a model. An evolutionary mechanism that altered the function of the Δ11-desaturase (Löfstedt et al. 1986) in a common ancestor of the lineage leading to *Y. rorrella* and *Y. gigas* may explain the major shift that took place in pheromone blend composition prior to divergence of these two species from their closest ancestor with unsaturated pheromone components. Genetic changes with major effects on chain shortening, desaturation, and fatty acyl reduction have been postulated (Jurenka et al. 1994) or characterized (Roelofs et al. 2002; Lassance et al. 2010, 2013) in other species.

Y. *rorrella* was found to be almost completely monomorphic at some 75 enzyme loci for which its congeners exhibit a normal to high proportion of heterozygous loci (Menken 1987). The most likely explanation for this lack of variation would be a (series of) bottleneck(s) at the species origin, which would fit a scenario of saltational speciation involving relaxed selection or genetic drift on the sex pheromone during the bottleneck. *Y. gigas* also exhibits a low genetic variability (Menken 1987; Menken et al. 1992) although not as low as in *Y. rorrella*. This divergence should have taken place at least a million year ago and according to Menken (1987), it would not take more than 200,000 generations (years) to restore a normal level of genetic variation. Consequently, one or more bottlenecks in a common ancestor of the species pair *gigas*/*rorrella* in addition to one or more bottlenecks in the lineage giving rise to *Y. rorrella* at the time of divergence of *Y. rorrella* from its closest relative would best explain the current dearth of genetic variation (Menken 1992). The idea of speciation by genetic revolution and the passage through a bottleneck has

been criticized because theoretical models show that under many assumptions the probability is low for the transition of a founder population to a new selective equilibrium reproductively isolated from the ancestral population. Nor did the reports on an aberrant number of chromosome pairs in *Y. rorrella* (29 compared to 31 in other species) (Thorpe 1929; Gershenson 1967) that would have supported the hypothesis about a genetic revolution hold up to closer examination. Studies of the karyotype in *Y. rorrella* and five other *Yponomeuta* species revealed the same haploid chromosome number in all species: 29 autosomes and a sex chromosome trivalent in females and 30 autosomes and one pair of sex chromosomes in males (Nilsson et al. 1988).

Regardless of the specific mechanism, *Y. rorrella* has obviously lost unsaturated pheromone components present in its ancestors and closest relatives. Besides alterations in biosynthetic pathways, alterations on the receiver side must have occurred involving gradual or saltational changes. Further studies should focus on *Y. rorrella* as model species. The broad olfactory receptor response of conspecific males may indicate that rare males in ancestral *Y. rorrella* populations were likely able to respond to the saturated acetate. The possible refinement of one type of OSN toward 14Ac together with the evolution of an antagonistic response to unsaturated acetates in this species would support a scenario for saltational speciation (Baker 2002) through adaptive asymmetric tracking (Phelan 1997).

(B) Molecular and functional aspects of pheromone production and reception. In light of the aforementioned observations, comparative approaches to fully elucidate the molecular and functional aspects of pheromone production and reception are still needed to contribute to a better understanding of the role of pheromones in speciation. The identification of two biosynthetic genes implicated in female signal production at the molecular level has started to shed light on some key enzymes shaping the production of multicomponent pheromones in *Yponomeuta*, but how species-specific blend ratios of alcohols and acetates are modulated in each species remains to be investigated. For this, a detailed comparative analysis of desaturase and reductase activities in the nine species is needed, together with a breakthrough in the characterization of chain shortening and acetyl-transferase enzymes. This would improve our understanding of the polygenic and functional nature of species-specific blend ratio formation among closely related species. Investigating the proximate mechanisms of pheromone reception by characterizing the number of receptors and assessing their functionality and specificity in vitro (e.g., Wanner et al. 2010; Zhang and Löfstedt 2013) will also further contribute to deciphering the evolutionary mechanisms that have shaped male-specific sensory adaptations in small ermine moths and contribute to our understanding of the potential adaptive role of male responses in the evolution of new species (Roelofs et al. 2002; Baker 2002).

(C) Ecological and evolutionary forces toward new signals. Little is known about the circumstances that have favored the maintenance and fixation of new pheromone signals. The pheromone differences observed among synchronic species with overlapping geographical distribution are consistent with reproductive character displacement. Selection for pheromonal differences, in particular divergence in Z/E11-14Ac ratios and antagonistic male responses between divergent populations, could have promoted unique communication channels to avoid interspecific hybridization and have at least facilitated coexistence of the three species (*Y. cagnagella*, *Y. plumbella*, and *Y. irrorella*) that share the European spindle tree as host plant by reducing communication interference. For other species, however, divergence by geographic isolation and drift cannot be ruled out as alternative explanations for pheromone specificity. Further evidence is therefore needed to provide conclusive answers as to which circumstances and ecological forces have favored the evolution of new signals in the genus *Yponomeuta*. Nothing is known about the evolution of the matching changes that are necessary at the level of the receiver for the evolution of a new preference. With the molecular, functional, and bioinformatic tools now available, such future comparative studies will better integrate pheromone production and reception to open the door for a continuation of the "small ermine moth project" in the (post) genomic era to understand how matching changes have come about in signal and response in *Yponomeuta* species.

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