Chasing sympatric speciation: The relative importance and genetic basis of prezygotic isolation barriers in diverging populations of Spodoptera frugiperda

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GENERAL INTRODUCTION

SPECIATION IN SYMPATRY

'...whilst this planet has gone cycling on [...], from so simple a beginning endless forms most beautiful and most wonderful have been, and are being, evolved.'
Charles Darwin, *On the Origin of Species*, 1859

When Darwin introduced the idea of speciation in his famous book *On the origin of species* (1859), his theory included the evolution of species in sympatry as well as in allopatry. Sympatric speciation is the evolution of new species from an ancestral species without geographic isolation, whereas allopatric speciation is facilitated by geographical boundaries separating populations. Today it is widely accepted that also without spatial separation of populations gene flow may become limited between groups of individuals and allow reproductive isolation to evolve (recent studies include e.g. Nanda and Singh 2012; Nosil and Feder 2012; Strasburg et al. 2012; Boomsma and Nash 2014; Castiglia 2014; Scordato et al. 2014; Aboagye-Antwi et al. 2015). However, for over a century sympatric speciation was widely rejected and has been referred to as the 'ugly duckling' of evolutionary theory, which only in the past few decades grew up to be a 'swan' (Via 2001). Why has this theory been so controversial for so long? Ernst Mayr's decade-long wholehearted battle for the acceptance of allopatry as universal mechanism of speciation, and his influential opinion was instrumental in the rejection of sympatric speciation (Berlocher and Feder 2002). Ernst Mayr was convinced that gene flow is unavoidable between populations that are not spatially separated, and populations cannot genetically differentiate in the face of gene flow (Mayr 1947). Additionally, empiric evidence for speciation in sympatry was rare. One prominent example had already been discovered in the 1860s: Benjamin Walsh had observed that in the apple maggot fly (*Rhagoletis pomonella*) host-races had formed by shifting from native hawthorn (*Crataegus* spp.) to introduced domesticated apple (*Malus pumila*). Based on this observation, he proposed that phytophagous insect species may evolve in sympatry by shifting and adapting to new host plants (Walsh 1864, 1867). In the debate initiated by Mayr almost a century later, Mayr's former student Guy L. Bush picked up Walsh's studies from the 1860s and re-introduced *Rhagoletis pomonella* as potential case of sympatric speciation (Bush 1969). In 1988, Bush's concept of sympatric speciation in *Rhagoletis* was substantiated by the confirmation of genetic differentiation between the two host-races (Feder et al. 1988; McPheron et al. 1988). The host-shift of *Rhagoletis pomonella* has since become a role model for speciation in sympatry in phytophagous insects and subject to extensive research into the mechanisms involved in the shift (e.g. Feder et al. 1994; Nojima et al. 2003; Olsson et al. 2006). Today, many additional examples of diverging sympatric populations are known and investigated, for example the pea aphid (*Acyrthosiphon*

pisum) (Via 1999; Via et al. 2000), the Goldenrod ball-gall fly (*Eurosta solidaginis*) (Abrahamson and Weis 1997) and cichlid fishes (Cichlidae) (Schliewen et al. 1994; Danley et al. 2000; Wilson et al. 2000).

The extensive debate of the past decades forged a better picture of the circumstances favorable for sympatric speciation, especially when mediated by a host-shift. Among these circumstances are a broad sympatric overlap of host patches, limitation of gene flow by differing habitat choice and a genetic basis for habitat choice (Via 2001). Additionally, multiple selective forces rather than one single force seem to be necessary to drive speciation in sympatry (Rice and Hostert 1993; Via 2001).

Despite a better understanding of possible mechanisms that may cause sympatric speciation that was accumulated over the past decades (Smadja and Butlin 2011), quite a few questions still remain: Does sympatric speciation depend on specific starting criteria and can these criteria be generalized? Can the sequence in which different reproductive isolation barriers evolve in sympatry be predicted and is this comparable to the sequence of events in allopatric speciation? How rapidly must reproductive isolation evolve for speciation in sympatry to occur (Via 2001)? To answer these questions it is important to identify the causes and consequences of reduced gene flow in species where reproductive isolation has not yet been completed, i.e. in diverging races with different modes of reproductive isolation, which are partially isolated in the present rather than investigating fully isolated species (Berlocher and Feder 2002; Drés and Mallet 2002; Via 2002; Via and West 2008).

The fall armyworm *Spodoptera frugiperda* is one of the invaluable case studies with two diverging strains in sympatry. The two strains are hypothesized to currently be in an incipient stage of sympatric speciation and exhibit three major potential isolation barriers: differential host plant usage, differential sexual communication and differentiation in daily rhythms (Groot et al. 2010). Thus, these strains constitute an ideal model system to study a) incipient sympatric speciation and b) the contributions and interactions of different isolation barriers to speciation in sympatry.

THE MODEL SYSTEM AND AIM OF THIS THESIS

This thesis aims to investigate the relative importance of the different potential prezygotic isolation barriers for the divergence of the two strains of *S. frugiperda* in sympatry, by identifying the molecular differences underlying these isolation barriers, so that possible interactions between the isolation barriers can be determined. Identifying the mechanisms underlying prezygotic isolation barriers in two sympatrically occurring strains of a species will give important insight in the first steps of sympatric speciation.

Biology of Spodoptera frugiperda

Spodoptera frugiperda (Lepidoptera: Noctuidae) is a noctuid moth inhabiting in North- and South America (Sparks 1979). As a generalist, its larvae feed on a huge variety of plants. Exactly how many different plants are fed upon by *S. frugiperda* larvae is unknown, but plants of 80 different species in 23 plant families have been described so far (Luginbill 1928; Pashley 1988). Among the host plants are many important crop plants, such as rice (*Oryza sativa*), corn (*Zea mays*), sorghum (*Sorghum bicolor*) (all Poaceae) and cotton (*Gossypium hirsutum*, Malvaceae).

Life cycle

Dependent on the climate, *S. frugiperda* populations may occur the whole year in warm climate, overwinter as pupae in mild winters (Sparks 1979) or die in the cold season and repopulate an area in the next warm season through migration (Nagoshi et al. 2008b, 2012). One life cycle is completed in 4-13 weeks, depending on the temperature (Sparks 1979; Andrews 1988) and can be summarized as follows. Eggs are usually laid in clutches of up to hundreds of eggs which are multilayered and can be covered with scales (Meagher et al. 2011) (Figure 1A). In fertilized eggs, the head capsule of the developing larva becomes visible within 2-4 days after oviposition and the eggs appear black (Figure 1B). Neonate larvae hatch from the eggs approximately 1 day later (Figure 1C). Larvae develop on their host plants during six instars, growing continuously and shedding their cuticle between instars (Figure 1D, E). Sixth instars dig 1-3 inches deep into the soil, where they pupate (Figure 1F). The adults eclose underground after 1-5 weeks and then leave the soil to unfold their wings (Figure 1G). The adult moths are usually not reproductively active before the second night of their adulthood. Then, females sit as high as possible on a host plant, extrude their pheromone glands and emit a sex pheromone attractive to males (female calling, Figure 1H1). Males are attracted from a long range and in close range show a specific male calling behavior: They extrude hair pencils from their abdomen, perform wing fanning and attempt to mate with the female by bending the abdomen towards her (Figure 1H2). Several males can approach one female, and both females and males mate with one partner per night. With whom the female mates is probably mediated by close-range communication via a male pheromone (Birch et al. 1990). Once mated, the copulation can extend over several hours (Sparks 1979; Schöfl et al. 2009) (Figure 1I). Females can mate with different partners in consecutive nights, and eggs may be fertilized by sperm of several males (Meagher and Nagoshi 2010). The female oviposits for the first time in the night following the first successful copulation (thus at the earliest in the 3rd night of adulthood) and eggs are placed on host plants as well as on non-host plants and even man-made objects like car tires or window panes (R.L. Meagher, pers. comm.). This wide distribution of eggs may be important for larval dispersal, but could also be a strategy of predator and parasitoid avoidance (Meagher et al. 2011).

FIGURE 1. Life cycle of *Spodoptera frugiperda*. (A) Fresh egg clutch. (B) Mature egg clutch with head capsules of larvae visible. (C) Hatching neonate larvae. (D) Early instar larvae. (E) Late instar larva. (F) Pupa. (G) Adult moths, male left and female right. (H1) Female calling. (H2) Abdomen of calling male, arrow points to extruded hair pencils. (I) Mating couple. (J) Female oviposition.

Strain differentiation

Spodoptera frugiperda occurs as two morphologically indistinguishable, but genetically differentiated strains. These two strains were originally identified by allozyme analysis of *S. frugiperda* specimens sampled from corn plants in Louisiana and Puerto Rico, and rice plants in Puerto Rico and Bermuda grass plants in Louisiana (Pashley et al. 1985; Pashley 1986). Pashley found host-plant specific differentiation at five loci and proposed the existence of at least two sibling species that thus were reproductively isolated (Pashley 1986). Numerous studies followed

these findings and identified a variety of additional molecular markers that show strain-specific differentiation (summarized in Table 1).

TABLE 1. Summary of studies that identified strain-specific molecular markers or using these markers to further elucidate the strain-differentiation.

Genomic DNA: esterase allozymes	Pashley 1986
Mitochondrial DNA: cytochrome oxidase 1 (CO1) and NADH dehydrogenase 1 (ND1)	Pashley 1989, Pashley and Ke 1992, Lu and Adang 1996, Levy et al. 2002, Meagher and Gallo-Meagher 2003, Nagoshi et al. 2006, Lewter et al. 2006, Lewter et al. 2007, Machado et al. 2008, Juárez et al. 2012, Dumas et al. 2015a
Genomic DNA: FR tandem repeat sequence (present in rice-strain, absent in corn-strain)	Lu et al. 1994, Nagoshi and Meagher 2003, Nagoshi et al. 2008a
Genomic DNA: Amplified fragment length polymorphisms (AFLP)	McMichael and Prowell 1999, Busato et al. 2004, Prowell et al. 2004, Clark et al. 2007, Martinelli et al 2007, Juárez et al. 2014
Genomic DNA: Triose phosphate isomerase (*tpi*)	Nagoshi 2010, Juárez et al. 2014
Genomic DNA: Microsatellite markers	Dumas et al. 2015b

Currently, differences in the mitochondrial cytochrome oxidase 1 (CO1) gene are mainly used to discriminate the two strains, as this is a comparably fast and inexpensive method: a 600 bp amplicon of the gene is digested with *Sac*I and *Msp*I restriction enzymes. The corn-strain amplicon has only the *Msp*I restriction site, whereas *Sac*1 digests only the rice-strain amplicon. As mitochondria are maternally transmitted, hybrids can only be identified by additionally using a diagnostic nuclear marker, e.g. the triose phosphate isomerase gene (*tpi*) (Nagoshi 2010). With a combined analysis of mitochondrial and nuclear markers, up to 16% inter-strain hybrids have been detected in field populations (Nagoshi and Meagher 2003a; Prowell et al. 2004; Nagoshi et al. 2006b; Machado et al. 2008). While these high rates of hybridization suggest incipient rather than completed speciation, recent studies based on microsatellite markers and CO1 sequences indicate that the two strains are actually 'good' species (Dumas et al. 2015a,b). However, for the sake of consistency with the majority of fall armyworm publications, I will use strains throughout my thesis.

Besides molecular differences, the strains show differentiation in their host utilization (Pashley et al. 1985; Pashley 1986, 1988, 1989; Meagher and Gallo-Meagher 2003; Prowell et al. 2004; Nagoshi et al. 2006a; Machado et al. 2008), composition of female sex pheromone (Groot et al. 2008; Lima and McNeil 2009; Unbehend et al. 2013) and timing of reproductive activity (Pashley et al. 1992; Schöfl et al. 2009). These three prezygotic isolation differences will be introduced

in detail in part 3 of this introduction. In addition to the prezygotic isolation barriers, the strains show some postzygotic isolation. In laboratory experiments, RC hybrid (offspring of rice-strain ♀ and corn-strain ♂) females showed reduced fertility (Pashley and Martin 1987; Whitford et al. 1988; Groot et al. 2010). Interestingly, the majority of hybrids found in the field are RC hybrids (Nagoshi and Meagher 2003a; Prowell et al. 2004; Nagoshi et al. 2006b), which may thus partly explain the reduced gene flow between the strains. Recently, Kost et al. (2015) found that the reduced fertility of RC hybrid females is due to such females being sexually abstinent, i.e. they do not mate with any mating partner (R, C, RC or CR males). This unidirectional postzygotic isolation barrier, in combination with the prezygotic isolation barriers, probably make up the reproductive isolation syndrome in the *S. frugiperda* strains (Groot et al. 2010; Kost et al. 2015).

Prezygotic isolation between the two* S. frugiperda *strains

Spodoptera frugiperda larvae cause annual crop losses of up to millions of US dollars (Wiseman et al. 1983), but fall armyworm research extends beyond developing monitoring or pest management strategies (Mitchell et al. 1985; Sparks 1986; Andrews 1988; Hruska and Gladstone 1988; Pitre 1988; Wiseman and Isenhour 1988a,b; Andrade et al. 2000; Malo et al. 2001; Vergara and Pitre 2001; Molina-Ochoa et al. 2003a,b; Hoballah et al. 2004; Bueno et al. 2008). The fact that the species occurs as two genetically differentiated strains in sympatry has initiated a broad array of studies from different perspectives, which address the central question: 'What keeps the strains apart?'

Three main prezygotic isolation barriers have been identified in *S. frugiperda*: Habitat isolation, behavioral isolation through strain-specific sexual pheromone communication and behavioral isolation through strain-specific timing of reproduction.

Habitat isolation through strain-specific host utilization

The best investigated isolation barrier that drives divergence between sympatric herbivorous insects is habitat isolation. When two insect populations mainly utilize different host plant species, this reduces the probability of these populations of mating with each other, resulting in reduced gene flow. Genetic differences between the populations can thus accumulate in the populations, differentiation into host race formation, eventually enabling reproductive isolation and the appearance of new species (Schluter 2001; Drés and Mallet 2002; Funk et al. 2002, 2006; Coyne and Orr 2004; Rundle and Nosil 2005; Feder et al. 2012). Host races constitute an intermediate step in ecological speciation in sympatry, which is a continuous process from polymorphisms between populations of the same species to fully distinguished species (Berlocher and Feder 2002; Drés and Mallet 2002).

Research addressing ecological speciation in sympatry is mainly focused on specialist herbivorous insects, like the apple maggot fly *Rhagoletis pomonella*

(Bush 1969; Feder et al. 1994), the Goldenrod ball-gall fly *Eurosta solidaginis* (Craig et al. 1993; Craig and Itami 2011) and the treehopper *Enchenopa binotata* (Wood 1980; Guttman et al. 1981). However, there are also examples of generalist herbivorous insects that show host use differences between populations: in the European corn borer, *Ostrinia nubilalis*, the pheromone Z strain feeds on maize, whereas the E strain primarily feeds on mugwort, at least in France (Thomas et al. 2003; Bethenod et al. 2005); in the larch budmoth *Zeiraphera diniana* one biotype prefers larch (*Larix* spec.), whereas another biotype prefers pine (*Pinus* spec.) (Emelianov et al. 1995, 2003, 2004), and in the tobacco budworm *Heliothis virescens* two populations have been recently recognized that perform differently on chickpea (*Cicer arietinum*) and cotton (*Gossypium hirsutum*) (Blanco et al. 2008; Karpinski et al. 2014). If these differences have a genetic basis that can be selected for (Emelianov et al. 2003; Thomas et al. 2003; Karpinski et al. 2014), differential host plant choice could start the process of ecological speciation in sympatry.

Spodoptera frugiperda may be one more example of a generalist herbivorous insect undergoing sympatric speciation through habitat isolation. After Pashley's first identification (Pashley 1986), larvae collected from tall grasses like corn and sorghum (*Sorghum bicolor*) and from cotton were up to 80% corn-strain individuals, whereas larvae collected from smaller grasses, like rice or pasture (e.g. bermudagrass) were up to 95% rice-strain larvae (Pashley 1986, 1988, 1989; Meagher and Gallo-Meagher 2003; Prowell et al. 2004; Nagoshi et al. 2006a; Machado et al. 2008). In this thesis I further explore the level and extent of host plant differentiation of the two strains. An overview of the physiological and behavioural studies and their potential to explain the differential distribution in the field is given in **Chapter 2** (Hänniger et al. 2015a). In **Chapter 3** (Juárez et al. 2014), we determined the strains of specimens collected from different host plants in three South American countries, and found only a weak host-association for this large data set. In **Chapter 8** (Groot et al. 2015) we give an overview of the strain identities of various field collections reported in literature as well as our own collections. Main findings are that field observations, oviposition studies and larval performance studies overall show inconsistent results in terms of host preference and performance of the two strains, which leads to the question of how strong the host association of the two strains actually is and to what extent this contributes to reproductive isolation between the strains (discussed in **Chapter 9)**.

Behavioral isolation through strain-specific sexual communication

Besides host plant differentiation, the two strains of *S. frugiperda* exhibit differences in their sexual communication (Groot et al. 2008; Lima and McNeil 2009; Unbehend et al. 2013). In moths, the female uses species-specific pheromone signals to attract males over long distances (Tamaki 1985; Löfstedt and Kozlov 1997). When the female extrudes the pheromone gland from her abdomen,

pheromone is emitted (Tamaki 1985; Percy-Cunningham and MacDonald 1987). Males detect the female sex pheromone with their very sensitive antennae and in response start flying towards the female, following the pheromone signal (Baker et al. 1985; Mafraneto and Cardé 1994). At close range, males exhibit a typical male courtship behavior by extruding hairpencils from their abdomen and wing fanning (Tamaki 1985; Birch et al. 1990; Lassance and Löfstedt 2009). If both partners are attracted to each other, copulation will ensue.

The female sex pheromone is species-specific and thus usually only attracts conspecific mating partners (Tamaki 1985; Löfstedt and Kozlov 1997). This specificity is realized by the combination of specific pheromone components as well as a species-specific ratio of these components (Tamaki 1985; Jurenka 2004). A component of a sex pheromone in one blend of one species can be used in a lower concentration by a different species, e.g. the major compound in *S. frugiperda* pheromone, (Z)-9-tetradecenyl acetate (Tumlinson et al. 1986) is present in lower amounts in the pheromone of *S. litura* (Sun et al. 2002) without causing cross attraction. Some components may also repel closely related species to avoid attraction of co-occurring heterospecific males (Vickers and Baker 1997; Groot et al. 2006; Eizaguirre et al. 2007). As moths usually have a very short reproductive phase (generally < 2 weeks), it is essential to have a reliable sexual communication system that ensures mating success. If changes occur in either the female sender of a pheromone signal or the male receiver, this may initiate reproductive isolation (Löfstedt 1993; Cardé and Haynes 2004).

The most prominent example of behavioral isolation through differences in sexual communication in Lepidoptera is the European corn borer, *O. nubilalis*, with two pheromone strains, E and Z (Smadja and Butlin 2009; Wicker-Thomas 2011; Lassance et al. 2013). In the Z-strain, females produce a 3:97 ratio of (E)-11-tetradecenyl acetate to (Z)-11-tetradecenyl acetate, whereas E-strain females produce a pheromone ratio of 99/1 (E):(Z)-11-tetradecenyl acetate (Klun et al. 1973; Kochansky et al. 1975). The opposite pheromone ratio found in E- and Z-strain females is caused by the fatty acyl reductase *pg*FAR gene product (Lassance et al. 2010). As for male response, the Z-strain males have a narrow response-range, flying only towards a blend with a 3:97 ratio, whereas some E-strain males also respond to intermediate E/Z ratios and can even be attracted to a Z-strain female (Roelofs et al. 1987; Glover et al. 1990). Consequently, mainly hybrids between E-males and Z-females are found in the field (Liebherr and Roelofs 1975). Hybrid males respond to a broad range of E/Z-ratios, and rarely to the E-strain females (Roelofs et al. 1987; Glover et al. 1990). For the male response, a *resp* locus on the sex chromosome has been identified (Roelofs et al. 1987; Dopman et al. 2004), as well autosomal and sex-linked loci affecting the antennal response (Roelofs et al. 1987; Olsson et al. 2010). Thus, sexual communication constitutes a strong isolation barrier in *O. nubilalis*, which appears to undergo sympatric speciation through

sexual communication differentiation, and may already be sibling species (Cardé et al. 1978; Malausa et al. 2007; Lassance et al. 2010).

In *S. frugiperda*, at least two behaviorally active components constitute the female sex pheromone: the major sex pheromone component (Z)-9-tetradecenyl acetate (Z9-14:OAc) and the critical secondary sex pheromone component (Z)-7-dodecenyl acetate (Z7-12:OAc) that makes up only a few percent of the pheromone (Tumlinson et al. 1986). Interestingly, corn-strain females consistently exhibited lower relative amounts of Z7-12:OAc than rice-strain females in laboratory as well as field populations (Groot et al. 2008; Lima and McNeil 2009). We determined whether the different pheromone blends of the corn-strain and rice-strain females are differentially attractive to males from the same strain, in wind tunnel assays as well as in male trapping experiments (**Chapter 4,** Unbehend et al. 2013). We also determined whether sex pheromone differences as well as differences in the male response differ between geographic regions (**Chapter 5**, Unbehend et al. 2014). In addition, we conducted quantitative trait locus (QTL) analysis to determine the genetic basis of the pheromonal differences between the two strain (**Chapter 6**, Hänniger et al. 2015b).

Behavioral isolation through strain-specific timing of reproduction

The most pronounced difference between the two strains of *S. frugiperda* is their timing of reproductive activity at night (Pashley et al. 1992; Schöfl et al. 2009). Pashley et al. (1992) observed 16 pure strain matings and found the corn-strain to mate in the first six hours of the scotophase, whereas the rice-strain started to mate after the sixth hour into the scotophase. Repeating the experiment with a much larger sample size (320-400 matings), Schöfl et al. (2009) confirmed that the corn-strain mates significantly earlier than the rice-strain. If populations of the same species are (reproductively) active in different time-windows at night, this could constitute reproductive isolation and thus may drive speciation in sympatry. Allochronic speciation in insects has been suggested for crickets (Alexander and Bigelow 1960; Danley et al. 2007; Fergus et al. 2011; Fergus and Shaw 2013) as well as fruit flies (Tauber et al. 2003; Prabhakaran and Sheeba 2012) and mosquitoes (Rund et al. 2012). Such temporal differences may be seasonal (e.g. *Laupala*) or within a day (e.g. *Anopheles gambiae*), both narrow the possible time windows for mating between individuals with different time windows. Surprisingly little is known about the genetic changes underlying these timing differences (reviewed in Groot 2014). Candidate genes that could underlie changes in daily rhythms are genes involved in the circadian clock. The circadian clock is a complex network of genes and their products, which enhance and suppress each other in a rhythmic manner, and which are entrained by environmental cues, such as light, temperature and/or tides. These molecular networks and their evolution have been subject of extensive research since the 1960s (Aschoff 1960; Pittendrigh 1960,

1961, 1993) and we are now beginning to understand how these molecules interact and have evolved to form biological clocks in the different kingdoms of life (e.g. Hardin 2005, 2011; Zhan et al. 2011; Hermann et al. 2013).

Insect circadian clocks have been extensively studied in *Drosophila melanogaster* and also in the Monarch butterfly, *Danaus plexippus* (Zhan et al. 2011). In *D. plexippus*, as in *Drosophila* (Hardin 2005), proteins (written in upper case letters, e.g. CLOCK) and genes (in italicized lower case letters, e.g. *cycle*) form two interlocked feedback loops, connected by their mutual usage of CLOCK (CLK) and CYCLE (CYC). In the main feedback loop, the transcription of *period* (*per*), *timeless* (*tim*) and *cryptochrome 2* (*cry2*) is promoted by a heterodimer of CLK and CYC binding to E-box elements in the promoter of these genes. PER, TIM and CRY2 proteins co-locate and enter the nucleus together, where CRY2 inhibits the CLK:CYC mediated transcription, including its own transcription. Light-entrainment of this feedback loop is facilitated by the blue-light receptor CRYPTOCHROME 1 (CRY1) promoting TIM degradation. SUPERNUMERARY LIMBS (SLIMB) and JETLAG (JET) signal the degradation of PER and TIM, proteins involved in CRY2 degradation have not yet been identified. Kinases (e.g. CASEIN KINASE II (CKII) and DOUBLE-TIME (DBT)) and phosphatases (e.g. PROTEIN PHOSPHATASE 2A (PP2A)) are involved in the post-translational modification of PER and TIM (Zhan et al. 2011). In *Drosophila*, PER instead of CRY2 inhibits the CLK:CYC promoted transcription and only one cryptochrome, homologous to CRY1, is present (Hardin 2005). When in *Drosophila* PER is degraded with the onset of the photophase, CLK:CYC dimers are freed to promote the transcription of *per*, *tim* and *cry2* and restart the main feedback loop. Additionally, the CLK:CYC dimers promote the transcription of *vrille* (*vri*) and *PAR-domain protein 1* (*pdp1*), starting the second feedback loop (Hardin 2005). In *D. plexippus*, like in *Drosophila*, *vri* inhibits *clk* transcription by binding to a V/P-Box in the clock promoter. *Pdp1* counteracts *vri* by promoting *clk* transcription (Cyran et al. 2003; Hardin 2005; Zhan et al. 2011).

Spodoptera frugiperda is an ideal model organism to investigate a) the genetic basis of allochronic differentiation and b) the influence of allochronic differentiation as isolation barrier between the two strains. In **Chapter 6** we present the results of a QTL analysis addressing the strain-specific timing of reproductive activity. We also show strain-specific expression differences and sequence polymorphisms of the major candidate gene, *vrille*. In **Chapter 7** we summarize the results of the annotation of clock genes in the genome of the corn-strain variant of *S. frugiperda*.

In **Chapter 9**, I discuss the main findings of this thesis. First, I discuss the potential of the different isolation barriers to facilitate reproductive isolation between the two strains of *S. frugiperda*. Secondly, I discuss possible interactions of the isolation barriers and their potential to drive the reproductive isolation between

the strains. I propose an interaction of all prezygotic mating barriers to facilitate the divergence of the two strains, with allochronic differentiation being the strongest force in these interactions.

In conclusion, by determining the level and extent of the different prezygotic isolation barriers that exist between the two strains of *Spodoptera frugiperda*, I have furthered our understanding of the mechanisms underlying the divergence between the strains. This example of incipient sympatric speciation can contribute to a better understanding of speciation in the face of gene flow.

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