Reproductive isolation and chemical communication in grasshoppers

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Abstract

The formation and maintenance of species in nature is accompanied by the evolution of reproductive isolating mechanisms. The identification and quantification of these reproductive isolation barriers is crucial to understand patterns of speciation and coexistence. In this thesis, I first conducted several experiments to identify and quantify reproductive isolation at multiple stages in the life history of the closely related species *Chorthippus biguttulus* and *C. mollis* (chapter 2). My results indicated a crucial role of chemical cues in the maintenance of species isolation. I combined multiple approaches to examine the ultimate and proximate causes of chemical cues on reproductive behavior in these species. In chapter 3, I demonstrated that the cuticular hydrocarbon (CHC) profiles of *C. biguttulus* and *C. mollis* provide species- and sex-specific cues. I used a RNA-seq approach to examine transcriptional differences of candidate genes, which might cause the divergence in CHC profiles between species and sex. One candidate gene showed species-specific transcriptional differences and may contribute to reproductive isolation. In addition, four candidate genes were differentially expressed between the sexes. Two of them exhibited a strong male-biased expression, which may be linked to higher proportions of dimethyl-branched CHCs in males. I found no evidence for positive selection acting on these genes, suggesting that differences in CHC profiles are presumably mediated at transcriptional level. In chapter 4, I developed a bioassay to determine if female CHCs act as chemical cues that induce courtship behavior in males, and if males assess variation in CHCs to determine whether or not to court a female. In summary, this thesis demonstrated that various reproductive isolating mechanisms reduce the gene flow between *C. biguttulus* and *C. mollis* and that in these species the courtship display consists of multimodal signals. In addition, my results suggest a key role of chemical cues in reproductive isolation and speciation.

Zusammenfassung

Der Prozess der Aufspaltung einer Art in zwei reproduktiv isolierte Arten in der Natur wird durch die Entstehung von Isolationsmechanismen begleitet. Die Identifizierung und Quantifizierung dieser Isolationsbarrieren ist wichtig, um die Muster von Artbildungsprozessen und die Koexistenz von zwei Arten zu verstehen. In dieser Arbeit identifizierte und quantifizierte ich zunächst mehrere Isolationsbarrieren zwischen den nah verwandten Feldheuschreckenarten *Chorthippus biguttulus* und *C. mollis* (Kapitel 2). Meine Ergebnisse deuten auf eine wichtige Rolle von chemischen Signalen bei der reproduktiven Isolation zwischen diesen Arten hin. Durch die Kombination von verschiedenen Ansätzen untersuchte ich die ultimaten und proximaten Ursachen von chemischen Signalen auf das Fortpflanzungsverhalten. Im dritten Kapitel zeigte ich, dass die kutikulären Kohlenwasserstoff Profile (CHC) von *C. biguttulus* und *C. mollis* art- und geschlechtsspezifisch sind. Mit Hilfe eines RNA-seq Ansatzes untersuchte ich transkriptionelle Unterschiede in Kandidatengenen, die für die Divergenz in den CHC Profilen zwischen den Arten und den Geschlechtern verantwortlich sein könnten. Ein solches Gen zeigte artspezifische Expression und trägt möglicherweise zur reproduktiven Isolation zwischen den Arten bei. Darüber hinaus fand ich Expressionsunterschiede zwischen den Geschlechtern in vier Kandidatengenen. Zwei von diesen Genen zeigten eine erhöhte Expression in Männchen, was eventuell in Verbindung mit dem höheren Anteil von dimethyl-verzweigten Kohlenwasserstoffen in Männchen steht. Ich fand keine Hinweise für positive Selektion in den Kandidatengenen, was vermuten lässt, dass die Unterschiede in CHC Profilen durch transkriptionelle Unterschiede entstehen. In Kapitel 4 erforschte ich mit Hilfe eines Bioassays, wie sich verschiedene weibliche und männliche CHC Signale auf das Balzverhalten von Männchen auswirkten. Zusammenfassend zeigt diese Arbeit, dass der Genfluss zwischen *C. biguttulus* und *C. mollis* durch verschiedene Barrieren unterbrochen ist und dass diese Feldheuschrecken multimodale Kanäle im Paarungsverhalten verwenden. Zusätzlich lassen meine Ergebnisse eine zentrale Rolle von kutikulären Kohlenwasserstoffen in der reproduktiven Isolation beider Arten und in der Artbildung vermuten.

Contents

List of Abbreviations

BDMI	Bateson-Dobzhansky-Muller-incompatibilities
BIMO	*C. biguttulus* ♀ x *C. mollis* ♂crossing
CHC	Cuticular hydrocarbons
CTRL	Control
C. big	*Chorthippus biguttulus*
C. mol	*Chorthippus mollis*
ELO	Elongase
FAS	Fatty acid synthase
F_{st}	Fixation index in genetic structure analysis (i.e., genetic distance value)
GCMS	Gas chromatograph mass spectrometer
NT	Not tested
MOBI	*C. mollis* ♀ x *C. biguttulus* ♂crossing
ORF	Open reading frame
PCA	Principal component analysis
RI	Reproductive isolation index
SD	Standard deviation
SE	Standard error

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1 General Introduction

"There is more to biology than rats, Drosophila, Caenorhabditis, and E. coli."[1]

Ernst Mayr criticized the prevalence of biological studies on the four major (genetic) model organisms. However, this quotation can also be understood as raising the question why there are more than just a few species? What are the reasons for species divergence and the origin of new species?

A common perspective in evolutionary biology is that speciation is a result of genetic divergence between populations accompanied by the evolution of reproductive isolation (Howard & Berlocher 1998; Schluter 2000; Coyne & Orr 2004; Gavrilets 2004; Butlin *et al.* 2009). Understanding a specific instance of speciation scenario requires a comprehensive understanding of the existence, strength and potential interactions of reproductive isolating barriers (Coyne & Orr 2004; Weissing *et al.* 2011; Seehausen *et al.* 2014). A barrier causes reproductive isolation when two populations produce no offspring or the offspring are less viable or fertile than expected from their relative abundance of the two populations in a given locality (Dobzhansky 1937; Mayr 1942; Coyne & Orr 2004). Reproductive isolation barriers can be divided into barriers which occur before zygote formation and thus prevent fertilization of eggs (prezygotic barriers) and barriers which act after formation of hybrid zygotes and result in lower viability or fertility of the hybrid offspring (postzygotic barriers). In many speciation scenarios it is often still unknown which barriers are most important in contributing to reproductive isolation, and it is also often controversial which barriers were involved in the initial reduction of gene flow between two populations (Panhuis *et al.* 2001; Coyne & Orr 2004; Butlin *et al.* 2009; Nosil *et al.* 2009). In addition, it is important to understand the evolutionary drivers of these barriers.

My dissertation research focuses on the question: which barriers actually contribute to reproductive isolation and discusses the evolutionary drivers under which the divergence between two closely related gomphocerine grasshoppers has increased and ultimately led to the formation of isolation barriers.

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[1] In Lynn Margulis and Dorion Sagan *Acquiring Genomes: A Theory of the Origins of Species*, Basic Books, Inc. New York, USA 2002

Reproductive isolation barriers are preferably studied in sympatry, because their effects on preventing gene flow can be directly observed, whereas in allopatry they can only be inferred (Coyne & Orr 2004). The reduction of gene flow between populations/species initiated by a barrier often depends on the interaction of the genotype with the environment. For instance, barriers that affect the co-occurrence of two populations/species reduce the access of an individual to potential mating partners. Thus, the strength of isolation depends on the environment in a wider sense, in which mating partners are part of the external environment (Seehausen *et al.* 2014). In addition, behavioral isolation (i.e., individuals prefer to mate with conspecific individuals) and extrinsic postzygotic isolation barriers (i.e., a lower mating success of hybrids) depend also on the interaction with other individuals (Seehausen *et al.* 2014). In contrast to prezygotic and extrinsic postzygotic isolation barriers, intrinsic postzygotic isolation relies exclusively on genotype-genotype interactions and is independent of the environment (Coyne & Orr 2004; Seehausen *et al.* 2014). In speciation scenarios where reproductive isolation depends on genotype-environment interaction it is often challenging to exclude or separate different evolutionary forces, for instance natural and sexual selection. Thus the role of different evolutionary forces on reproductive isolation and speciation can be best studied in systems where reproductive isolation is based on few rather than many barriers.

Grasshoppers are particularly suitable to study reproductive isolating mechanisms between species and populations and the underlying evolutionary forces, because they are widely distributed all over Europe and often occur sympatrically in high densities (Heller *et al.* 1998). Closely related grasshopper species are often primarily distinguishable by distinct mating signals, whereas differences in phenology, morphology or ecology are minor. Although, small changes in ecological preferences, like microhabitat adaptation or changes in temporal preference can occur even between closely related grasshopper species (reviewed in Ingrisch & Köhler 1998). Nevertheless, isolation based on barriers which affect the co-occurrence of two populations/species, such as temporal preference or habitat isolation, do not contribute to a great extent to total isolation and are not considered to initiate speciation alone. In addition the species are physically capable of mating with one another; hence there are no important barriers due to mechanical isolation of genital structure. In summary, grasshoppers are particular suitable to study the role of sexual selection and behavioral isolation on specia-

tion, since other barriers seemed to be either not present or less important for reproductive isolation.

Members of the grasshopper subfamily Gomphocerinae show an enormous diversity and complexity in courtship behavior, especially in the acoustic displays (Jacobs 1953; Perdeck 1958; Elsner & Wasser 1995; Greenfield 1997). Within grasshoppers (Acrididae) the subfamily Gomphocerinae has the highest species density (Heller *et al.* 1998). Recent radiations in this subfamily led to an expansion of species with weak genetic differentiation (Mayer *et al.* 2010; Vedenina & Mugue 2011). A taxonomical criterion of gomphocerine grasshoppers is the presence of a peg structure on the inner surface of the hind femur (Uvarov 1966). These grasshoppers produce acoustic signals by rubbing the pegs against the fore wings (Faber 1929; Jacobs 1953; von Helversen & Elsner 1977). The amplitude modulations of the signals are species-specific and serve for species recognition, mate localization and mate attraction (von Helversen & von Helversen 1983, 1997; Greenfield 1997; Hennig *et al.* 2004; Ronacher & Stange 2013). Acoustic signals are often an important taxonomical criterion distinguishing between closely related species (Ramme 1920; Faber 1929, 1953; Jacobs 1953). Female preferences for male acoustic signals were often used to draw conclusions about the strength of reproductive isolation between species (von Helversen & von Helversen 1975a,b, 1983; Gottsberger 2007). The ancestral form of mate attraction in grasshoppers consists of acoustic signals of males and phonotactic behavior of females, who use these signals to approach the sender (Heller *et al.* 1998; Gerhardt & Huber 2002). Some gomphocerine grasshopper species evolved a bidirectional communication system in which the female produces response songs that allow the male to approach the female by phonotaxis (Faber 1953; Elsner & Popov 1978; von Helversen & von Helversen 1997). The attractiveness of male songs and the shape of female preference function can be examined by using females' response probability to male songs (von Helversen 1972, 1997; von Helversen & von Helversen 1983). Evolutionary and neuroethological research in grasshoppers mainly focused on acoustic communication itself or sexual selection on acoustic signals (von Helversen & Elsner 1977; Ronacher & Römer 1985; Heinrich & Elsner 1997; Klappert & Reinhold 2003; Ronacher & Stange 2013). However, previous studies indicated that also non-acoustic cues are involved in mating behavior, but studies on other components than acoustic cues in mating behavior are underrepresented (Jacobs 1953; Ritchie 1990; Elsner & Wasser 1995; Butlin 1998; Tregenza *et al.*

2000b). In addition, interspecific crossing suggested that non-acoustic cues may also be important as a reproductive isolating mechanism.

In two of these hybridization studies only a few interspecific crosses were obtained over a period of two years (10 interspecific crosses in von Helversen & von Helversen 1975a, and 9 interspecefic crosses in Gottsberger 2007). Thus, the success rate of hybridization was very low, even though the females' resistance to heterospecific acoustic signals was circumvented during the crossing attempts by exposing the female exclusively to conspecific male songs during the experiment and by using as mates muted heterospecific males (von Helversen & von Helversen 1975a; Gottsberger 2007). The almost complete absence of hybrids in the field together with these results from interspecific lab crossing attempts implies that other isolation barriers, apart from acoustic signals, must operate. Gene flow between closely related species is expected to be reduced by multiple barriers that may act together (Coyne & Orr 2004). Theoretical models predict that speciation is facilitated when multiple sexual signals during courtship are present (Proulx & Servedio 2009; Doebeli & Ispolatov 2010). Although, prezygotic isolation is strong in grasshopper does not necessarily mean that later acting postzygotic (i.e., extrinsic and intrinsic) barriers were insignificant during speciation even they are weak (Coyne & Orr 2004). Thus, the prediction of a speciation scenario requires a precise knowledge about all reproductive isolating mechanisms within a species pair.

1.1 Scope of this thesis

This thesis focuses on the components and the mechanisms of reproductive isolation between the two closely related grasshopper species, *Chorthippus biguttulus* and *C. mollis*. For this purpose, I determined the sequential order of isolation barriers and estimated the strengths of prezygotic barriers as well as extrinsic and intrinsic postzygotic barriers. In addition, I investigated the ultimate and proximate causes of reproductive behavior using multiple methods and approaches, including behavioral testing, analytical chemistry and genetics.

In the second chapter, I describe several experiments to test the role of various components of reproductive isolation between *C. biguttulus* and *C. mollis*. I measured several life history traits of both parental species and I

produced hybrids and backcrosses in the laboratory to examine extrinsic- and intrinsic-postzygotic isolation. To test for prezygotic isolation (i.e., behavioral isolation) between the species pair, I recorded and analyzed acoustic mating traits. In addition, I performed behavioral experiments to examine the impact of acoustic signal on reproductive isolation. The interspecific crossing experiments suggested that others cues, in addition to acoustic cues, are important in courtship behavior and as isolation barriers.

In chapter 3, I tested the hypothesis that the cuticular hydrocarbon (CHC) profile of grasshoppers differs between species and sexes. To test this, I used a gas chromatography mass spectrography (GCMS) approach to explore whether there exists a general difference in CHC composition between *C. biguttulus* and *C. mollis* individuals. The same method was used to test how environmental conditions, such as rearing conditions and food source, affect the CHC profile. Subsequently, I searched for and identified candidate genes that are potentially involved in the generation and composition of the CHC profile. Finally, RNA-seq data were used to search for differential expression between species and sexes in these candidate genes and to calculate synonymous and nonsynonymous substitution rates for the sequences of candidate genes.

In chapter 4, I address the proximate question of how chemical signals may affect courtship behavior in males. Specifically, I developed a bioassay to test the male response to conspecific and heterospecific female odors. Furthermore, I tested the response of males to conspecific male odor and examined differences in response probability to female odor from distant populations.

Chapter 2, 3 and 4 address questions of different disciplines using specific methods. Therefore each chapter is structured in introduction, methods, results and discussion to introduce and discuss the specific subjects of each chapter in more detail. In the end, I discuss the implication of all results in a broader context and provide an outlook for further studies in the future.

2 Components of reproductive isolation between *C. biguttulus* and *C. mollis*

2.1 Introduction

Speciation can be driven by various factors of extrinsic selection, (e.g. divergent-, disruptive-, ecological selection) or by factors of intrinsic incompatibilities, such as genetic drift, genomic conflict, gene duplication (Coyne & Orr 2004; Seehausen *et al.* 2014). The identification of species isolating mechanisms is essential for understanding causes and consequences of evolutionary drivers and the origin of speciation processes (Dobzhansky 1937; Coyne & Orr 2004). A deep understanding of the present reproductive isolating mechanisms and their fitness consequences in a system is required to predict a specific speciation scenario (The Marie Curie Speciation Network 2012). However, it is often challenging to distinguish whether a barrier was involved in speciation process or evolved after speciation was complete. Thus, knowledge about the sequential order of a specific reproductive barriers in the life cycle of an organism is relevant to estimate its relative and absolute contribution to total isolation (Coyne & Orr 1989, 1997; Ramsey *et al.* 2003). In addition, the interactions of barriers can affect the selection pressure and can provide important information about the evolutionary drivers (Mendelson 2003; Coyne & Orr 2004). For instance, isolation barriers which affect the co-occurrence of species, like temporal or geographical differences affect the weight in gene flow estimation for following barriers (Sobel & Chen 2014). In general, reproductive isolation barriers are grouped into prezygotic barriers (e.g., difference in mating behavior, timing and location), extrinsic postzygotic barriers (e.g., offspring is behaviorally or ecologically isolated) and intrinsic postzygotic barriers (due to genetic incompatibilities)(Dobzhansky 1937; Mayr 1942).

Studies on reproductive isolation gomphocerine grasshoppers (Orthoptera: acrididae) have almost exclusively focused on prezygotic barriers and extrinsic postzygotic barriers (here where hybrid songs fail to be attractive to either parental species). These studies were primarily based on visual cues (Faber 1953; von Helversen 1986; Elsner & Wasser 1995), chemical cues

(Butlin, 1998; Ritchie, 1990; Tregenza et al. 2000b) and acoustic cues (Perdeck 1958; von Helversen & von Helversen 1975a,b; von Helversen 1997; Gottsberger & Mayer 2007). With a few exceptions, other isolation barriers were mostly not measured or quantified, (Hewitt *et al.* 1987a; Tregenza *et al.* 2002; Vedenina *et al.* 2007).

The speciation process in gomphocerine grasshoppers is assumed to be driven by hybridization and non-ecological divergence in allopatry (Mayer *et al.* 2010; Vedenina & Mugue 2011). These predictions are based on the assumption that species are reproductively isolated only on the basis on acoustic signals. However, several experiments suggests that also other cues contribute to reproductive isolation in gomphocerine grasshoppers (Ritchie 1990; Kriegbaum & von Helversen 1992; Butlin 1998). Estimation of the absolute and relative strength of the acoustic signal compared to other barriers is lacking.

Here I investigate the two gomphocerine grasshopper species, *C. biguttulus* and *C. mollis,* which have a bidirectional acoustic communication system. Males and females produce acoustic signals by rubbing their hind leg against their forewing (Faber 1929; Jacobs 1953; von Helversen & Elsner 1977). These signals differ strikingly between species and are evaluated by males and females (Figure 2.1). Both species are model system for acoustic communication research and widely used for studies of speciation, sexual selection and neuroethology (von Helversen & von Helversen 1975c, 1997; Kriegbaum 1989; Ronacher 1989, 1991; Kriegbaum & von Helversen 1992; Heinrich & Elsner 1997; Klappert & Reinhold 2003; Vedenina *et al.* 2007). Natural hybrids between these species have only rarely been found and hybridization experiments in the lab have revealed strong behavioral isolation (Perdeck 1958; von Helversen & von Helversen 1975a; Kriegbaum 1988). *Chorthippus biguttulus* and *C. mollis* are morphologically and genetically very similar and can occur sympatrically throughout Europe (Perdeck 1958; Ragge *et al.* 1988; Mason *et al.* 1995; Heller *et al.* 1998; Mayer *et al.* 2010).

The aim of this study is first to provide a comprehensive understanding of the present pre- and postzygotic reproductive barriers and their contribution to total isolation. These results will help to gain more insight into the underlying evolutionary forces that drove divergence between *C. biguttulus* and *C. mollis*. To achieve this, I conducted multiple experiments to measure the strength of prezygotic, extrinsic postzygotic and intrinsic postzygotic isolation barriers by producing F1 hybrids and Backcross generations in the la-

boratory. The reproductive isolation strength of each barrier was then estimated according to the sequential stage in the life cycle based on the method described by Sobel and Chen (2014). Further, barriers were combined by considering the potential interactions to estimate the total reproductive isolation and the absolute contribution of the studied barriers to total isolation (Sobel & Chen 2014). Based on the results I discuss different evolutionary forces which might have contributed to the origin of speciation between these species.

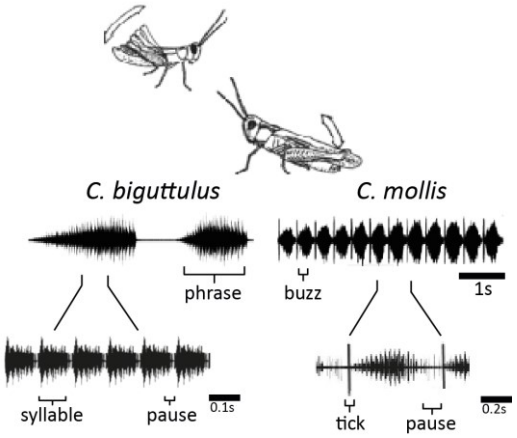

Figure 2.1 Illustration of *C. biguttulus* and *C. mollis* male song.
Lower sound traces show magnification of internal structure of the phrases and buzzes. Grasshopper illustration and lower sound traces are adapted from Helversen and Helversen (1994) and upper sound traces are adapted from Willemse et al. (2009)

2.2 Material and methods

Reproductive isolation barriers between *C. biguttulus* and *C. mollis*

Collection sites

In this study we used individuals collected in Mid-West (N51°28’10.41, E9°56’24.98), North-East (N52°32'3.33; E13°40'23.01) and South-East (N49°36’35.18, E10°59’3.05) Germany to produce F1 hybrids and Backcrosses. The population collected in Mid-West Germany is an allopatric *C. biguttulus* population, whereas the North-East and the South-East populations are sympatric for *C. biguttulus* and *C. mollis*.

Hybridization experiments

I use the term F1 hybrid for offspring produced from reciprocal crossings *C. biguttulus* and *C. mollis* during lab experiments. Field hybrids were never found for any location mentioned above during collection and excursions in the years 2012–2015. Hybridization experiments in the lab were strongly impeded by prezygotic barriers and only possible by deluding both sexes. For the heterospecific crossings we used a similar method as described by von and von Helversen (1975a) with minor adjustments. Heterospecific copulations were only successful when both male and female had experienced several conspecific copulation attempts (completion of which was prevented by the experimenter). After several attempts the male was then transferred during the next attempt and placed on a motivated heterospecific female. It was important that this female was also stimulated by several prior copulation attempts by a conspecific male, right before the transfer of the male. I observed no difference in the acceptance of heterospecific mating partners between the reciprocal F1 crosses. The offspring from a *C. biguttulus* female crossed with a *C. mollis* male were termed BIMO and the offspring from *C. mollis* females crossed with *C. biguttulus* males were called MOBI.

Backcross offspring were obtained by crossing a F1 hybrid female with a *C. biguttulus* male. The reciprocal cross was not successful, because all F1 hybrid males failed to fertilize *C. biguttulus* females (discussed below in the section intrinsic postzygotic isolation). Backcrosses to *C. mollis* were not produced due to a limited number of F1 hybrids. In the field the probability of a backcross individual to encounter a F1 hybrid individual of opposite sex is extreme low in view of the low fitness of F1 hybrids. Thus, I separated the calculation of reproductive strengths and provide the reproductive isolation

index (RI) of Backcrosses to the pure species in the main text and the RI values of Backcrosses to F1 hybrids in the Appendix A.

Measuring reproductive isolation

I identified and estimated the contribution of pre- and postzygotic barriers to reproductive isolation according to their sequential position in the life cycle. I used an Excel (Microsoft, Redmond, USA) spreadsheet provided by Sobel and Chen (2014) to calculate the reproductive isolation index (RI) and the cumulative strength of isolation. The RI calculation was based on the equation 4A described in Sobel and Chen (2014). The general form is

$$RI = 1 - 2 \times \left(\frac{heterospecific}{heterospecific + conspecific}\right),$$

where RI is the relative strength of a barrier, which can range between 1 at complete isolation, 0 at random mating and -1 when gene flow is facilitated (Sobel & Chen 2014). The term heterospecific is used for mixed-species pairing or Backcrosses, whereas the term conspecific is used for pure-species pairing. In addition I calculated the index of reproductive isolation of Backcrosses to F1 hybrids, in this case results of the F1 hybrids are classified as 'conspecific' and backcross values as 'heterospecific'.

The focal species are not temporally isolated, although slightly different population peaks are possible (see p.104 in Ingrisch & Köhler 1998). We have no evidence for reproductive isolation based on ecotype or microhabitat differentiation. Thus, I used a probability of 0.5 as the null expectation for an individual to meet or mate with conspecific or heterospecific individuals. The calculation of the cumulative strength of isolation was based on the equation 4E described by Sobel and Chen (2014)

$$RI_t = 1 - 2 \times \left(\frac{P(H|S) + P(H|U)}{S_{total} \times P(H|S) + U_{total} \times P(H|U) + S_{total} \times P(C|S) + U_{total} \times P(C|U)}\right)$$

with the assumption that there are no barriers which affect the co-occurrence of the two focal species, that means that shared (S) time and area between species is 1 and the unshared (U) time and area is 0. Thus, the cumulative strength between species was calculated as

$$RI_t = 1 - 2 \times \left(\frac{P(H|S)}{P(H|S) + P(C|S)}\right).$$

I estimated the probability of heterospecific gene flow in shared areas as

$$P(H|S) = \frac{S \times \prod_i^n (1 - RIi) \times 0.5}{\prod_i^n (1 - RIi) \times 0.5 + 1 - (\prod_i^n (1 - RIi) \times 0.5)}$$

and the probability of conspecific gene flow in shared areas as

$$P(C|S) = \frac{S \times \prod_i^n 1 - (1 - RIi) \times 0.5}{\prod_i^n 1 - (1 - RIi) \times 0.5 + \prod_i^n (1 - RIi) \times 0.5 + (1 - S)}$$

(Sobel & Chen 2014). The strength of postzygotic isolation alone was calculated as the cumulative strength of all postzygotic isolation under the assumption that no prezygotic barriers would exist (Appendix A).

Prezygotic barriers

Calling song preferences

To estimate the reproductive isolation strength for long range courtship behavior, I quantified female responses to male calling songs in playback experiments. The features of calling song models used to characterize the pure species in this thesis were based primarily on values used in previous studies of female preferences in these species (von Helversen & von Helversen 1975a,b, 1994, 1997). The typical *C. biguttulus* calling song model consisted of a 80 ms long noise "syllable" (5–40 kHz) of a constant amplitude followed by 15 ms of silence, repeated ca 32 times; the *C. mollis* calling song consisted of a 240 ms long noise syllable (2-40 kHz) of a constant amplitude followed by a 240 ms of silence, repeated ca 17 times. These songs mimic attractive characteristics of male songs and elicit reliable responses of conspecific females (von Helversen & von Helversen 1975b, 1994). The song duration was 3 s for the *C. biguttulus* and 8 s for the *C. mollis* song. For the experiment, a virgin *C. biguttulus* or *C. mollis* female was placed in a soundproof chamber. The stimulus playback and the recording of the female responses was controlled by custom–written software (Matthias Hennig, Humboldt Universität zu Berlin, Germany; for details about the experimental setup and testing procedure see (Schmidt *et al.* 2008; Reichert & Ronacher 2015). Females were exposed 18 times to each test stimulus in a randomized order. Only Females with more than 3 responses to the negative control (3 s of a continuous noise) were discarded as non–selective (*C. mollis* 4/26; *C. biguttulus* 0/46).

I used generalized estimation equations to test for significance between response probabilities of the conspecific and heterospecific test stimulus. The response variable was the number of female responses to a certain test stimulus, and this was modeled as a binomial variable (logit link function) by including a term for the number of times a female was exposed to a given stimulus. Individual female ID was included as random factor to account for the repeated measurements within females. The conspecific or heterospecific test stimulus was included as the explanatory variable. For analyzing the models, I used the *geeglm* function in the *geepack* package (Højsgaard et al. 2006) in R. The RI for *C. biguttulus* as conspecific species to *C. mollis* as heterospecific species was calculated by using the response probability of *C. biguttulus* females to a typical *C. mollis* song model as the 'heterospecific' value in the equation 4A and the response probability of *C. biguttulus* females to a typical *C. biguttulus* song model as the 'conspecific' value. The classification of 'heterospecific' and 'conspecific' was reversed for RI calculation with *C. mollis* as the conspecific species. For the RI calculation I used the average response probabilities from 46 *C. biguttulus* females and 22 *C. mollis* females.

Courtship behavior triggered by chemical cues

To explore the impact of chemical signals on reproductive isolation at close range, I used the results of a bioassay study, which quantified male choosiness on chemical cuticular cues (chapter 4). For RI calculation I calculated the true positive rate

$$TPR = \frac{\sum true\ positives}{\sum true\ positives + \sum false\ negatives}$$

of calling responses of *C. biguttulus* and *C. mollis* males to conspecific and heterospecific cuticular hydrocarbons (CHCs) of females based on Table 4.1 (chapter 4). Male TPR to heterospecific female CHCs was classified as 'heterospecific' and correspondingly the TPR of males to conspecific female CHCs as 'conspecific'.

Short range acoustic communication

To investigate the impact of the acoustic signal at close range, I quantified the mating success of intact males and muted males in *C. biguttulus*. I conducted two no choice experiments, one with 15 virgin *C. biguttulus* females and 10 intact *C. biguttulus* males (able to produce calling songs). For the second experiment I used 10 muted males instead of intact males. These males were

unable to produce calling songs since their fore wings were removed. This procedure has no influence on copulation attempts or viability of males (Perdeck 1958; Kriegbaum 1988). In both experiments females and males were kept in a mesh polyester cage (47.5 x 47.5 x 47.5 cm, bug dorm Taichung, Taiwan) together for 7 days at 25–30°C, 25–30% relative humidity, and a 16:8 h light-dark cycle. After this time the females were killed by freezing them for 30 min at -20°C. To estimate the mating success I dissected and transferred the sperm storage (*receptaculum seminis)* of females on a slide. I then squeezed the Receptaculum seminis with a cover slip to destroy the shell and to release the internal fluid. Females were counted as mated when sperm was found using a microscope (BH-2 Olympus, Tokyo, Japan) with 50x magnification. I examined statistical significance of test groups by using Fisher Exact test (*fisher.test*; R) on 14 females per test group (in each group one female died). For RI calculation I defined the mating success of mute males as 'heterospecific' and the mating success of intact males as 'conspecific' in the equation 4A.

Short range isolation between species in a no choice experiment

I quantified the mating success in a no choice experiment similar to the experiment described above with the difference that I used mute heterospecific males and examined the mating success after 7 days for 15 females for each species. The RI was calculated by using the proportion of mated females tested with muted conspecific males as 'conspecific' value in equation 4A and the proportion of mated females tested with muted heterospecific males as 'heterospecific' value in equation 4A.

Intrinsic postzygotic isolation

Even virgin females of *C. biguttulus* and *C. mollis* do produce eggs continuously and lay them as egg pods in repeating cycles (Kriegbaum 1997; Ingrisch & Köhler 1998). There is no evidence that egg laying cycles or egg production were affected by heterospecific matings. Thus, I used hatching success of offspring from heterospecific crossings as the first postzygotic reproductive isolation barrier.

Hatching success of pure species and F1 hybrids

The reproductive success of the different crossing types was assessed by examining the hatching success of the offspring. Breeding cages of the pure species contained a plastic cup filled with moist granulate (Vermiculite

Dämmstoffe, Germany) for oviposition. For heterospecific crossings each mating was initiated and observed by an experimenter to guarantee copulation success. After copulation, females were kept in separate cages also with a plastic cup for oviposition. The moist granulate was checked for egg–pods every second day. After oviposition egg pods were transferred to petri dishes filled with new moist granulate and were incubated for 30 days at 18–25°C, followed by an incubation in a fridge at 4°C for at least 90 days to ensure high hatching success (Ingrisch & Köhler 1998). To induce hatching, egg-pods were incubated at 20–25 °C until they hatched (12–28 days), after hatching the temperature was raised to 28–30°C. After 3 weeks of the last hatching event, I removed the egg–pods and counted empty egg shells and unhatched but fertilized eggs. I analyzed the differences in hatching success between crossing types with a logistic regression (*glm* with binomial distribution; R). I included the information about the crossing direction for the F1 hybrids and used a Tukey post hoc test (*glht* function from the package *multcomp*; R) to test for group differences, with p values adjusted for multiple comparisons using the Shaffer procedure. For the RI calculation with *C. biguttulus* or *C. mollis* as the conspecific species the hatching success of eggs produced by conspecific crossing were defined as 'conspecific' and the hatching success of eggs produced by reciprocal *C. biguttulus* x *C. mollis* crossing as 'heterospecific'. The same breeding design and methods were used to examine the hatching success of Backcrosses. The hatching success of backcrosses was then used as 'heterospecific' value in the equation 4A and the hatching success of *C. biguttulus* as 'conspecific'. For the comparison of Backcrosses with F1 hybrids, the hatching success of hybrids was used as the 'conspecific' and hatching success of Backcrosses as 'heterospecific' in equation 4A (Appendix A).

Survival rate of larvae

The survival of larvae was monitored every day between hatching and adult stage. The survival rate of larvae was then estimated for all groups as the percentage of larvae that succeeded in molting to the adult stage. In total, 146 *C. biguttulus*, 108 *C. mollis*, 70 BIMO, 133 MOBI and 61 Backcross larvae were analyzed. I used a Pearson's Chi-squared test (*chisq.test*; R) to test for differences between groups, followed by pairwise comparisons using the chisq.post.hoc function from the fifer package (Fife 2014), with p values adjusted for multiple comparisons using the FDR procedure. For the RI calcula-

tion the survival rates of larvae of *C. biguttulus* or *C. mollis* were defined as 'conspecific' and the survival rate of F1 hybrid larvae as 'heterospecific'. The survival rates of backcross larvae were compared to *C. biguttulus* and to F1 hybrid larvae (Appendix A).

Functional development of wing and hind leg morphology

To test whether hybridization affected the functional development of compartments which are essential for producing the acoustic signal, I examined the integrity of these compartments after the final molt. An individual which lacked one or both hind legs or showed disorders in wing morphology was counted as affected in the functionality of the sound producing organs. All grasshoppers were controlled within 24 hours after their final molt. The total sample size for each group was 34 for *C. mollis*, 11 for *C. biguttulus*, 100 for F1 hybrids and 29 for Backcrosses. Differences in functional development of acoustic organs between crossing types were analyzed with a logistic regression (*glm* with binomial distribution; R). For the RI calculation the disorder rate of *C. biguttulus* or *C. mollis* was defined as 'conspecific' and the disorder rate of F1 hybrids or Backcrosses as 'heterospecific'. For the reproductive isolation between Backcrosses and F1 hybrids the disorder rate of the F1 hybrids was used as the 'conspecific' value (Appendix A).

Internal sexual organs

To investigate potential effects of hybridization on the internal sexual organs of F1 hybrids, I counted the number of ovarioles of females and measured the testis weight in males. One egg matures per ovariole, thus the amount of eggs per egg–pod depends on the number of ovarioles. I used 17 *C. biguttulus*, 18 *C mollis* and 12 F1 hybrids females which were post mortem stored at -20°C in 70% EtOH until further processing. After dissection I counted the ovarioles. The ovariole numbers were not normally distributed and therefore I analyzed them using a Kruskal-Wallis test (*kruskal*; R).

In order to quantify the hybridization effects on males, I weighted the testes from 13 *C. biguttulus*, 13 *C. mollis* and 22 F1 hybrid (11 for each crossing type) males. Males were also stored post mortem in 70% EtOH at -20°C. After dissection and removal of the testes, testes were dried for 3h at 60°C in an incubator (T6060, Heraeus, Hanau, Germany) and then weighted on a micro scale (ALT 100-5AM, Kern & Sohn, Reproducibility = 0.03mg, Balingen, Germany). Testis weight correlates with body size, therefore I controlled for body size by measuring the femur size of one hind leg per individual (DeBano 2008).

The statistical analysis of the testis weight was performed by using an ANCOVA (*lm* and *anova* function in R). I controlled for testis weight effects due to body size differences by using testis weight as outcome variable, species group as predictor variable and femur length as covariate. The ANCOVA test predicts the independence of covariate and group, which was tested by using the *anova* function in R. The homogeneity of variance was tested with Levene's test (*leveneTest*; R). Subsequently, I used a post-hoc test to determine which groups differed significantly from each other (*TukeyHSD*; R).

Extrinsic postzygotic isolation

The category pre- or postzygotic to which a barrier belongs is described relative to the hybridizing species pair. For instance a lower courtship motivation of female hybrids relative to the parental species acts at the same prezygotic stage of an individual's life cycle but reduces the gene flow between hybridizing species after fertilization (postzygotic) in the same way as reduced hatching success of hybrid offspring does.

Courtship motivation of F1 hybrid and Backcross females

I estimated the courtship motivation level of females by quantifying the calling response probabilities of females to a repetition of one single test stimulus which was assessed as attractive by the female. Females were exposed to attractive conspecific artificial male song models and once the female started to respond to that test stimulus I examined the response probability of the female for eleven responses to that stimulus after her first response. I assumed that a motivated female would continuously respond to an attractive stimulus. These data were extracted from the calling song preference tests (for details of method and test stimulus design of pure species see 'Calling song preference'). The preference functions of F1 hybrid females are supposed to be similar to the parental species and not intermediate (von Helversen and von Helversen, 1975b), therefore I exposed F1 hybrid females to both pure species test stimuli. Once a F1 hybrid female responded to either of one of them, the female was only tested with the chosen one.

All the hybrids were backcrossed to *C. biguttulus*, thus all of the backcross females were tested with a typical *C. biguttulus* test stimulus. I performed Kruskal-Wallis tests, because data were not normally distributed. Pairwise comparisons between the groups were conducted using the posthoc Kruskal Nemenyi test function from the *PMCMR* package (Pohlert 2015). For

the RI calculation the motivation level (response probability) of *C. biguttulus* or *C. mollis* to conspecific test stimuli was defined as 'conspecific' and the motivation level of F1 hybrid females and Backcross females as 'heterospecific'. In addition, I calculated the RI for the motivation level of F1 hybrid females as 'conspecific' and the motivation level of Backcrosses as 'heterospecific' (Appendix A). For the calculation the average response probabilities from 27 *C. biguttulus*, 22 *C. mollis*, 32 F1 hybrids and 9 backcross females could be used.

Behavioral Isolation of F1 hybrid and Backcross males

To estimate the behavioral isolation strength of F1 hybrids males and Backcross males, I used song recordings to quantify in playback experiments the response probabilities of females to these recordings. Males were recorded separately, acoustically isolated from each other in a sound chamber (for details see Stange & Ronacher, 2012). The temperature during the recordings was maintained at 29 ± 2°C. I analyzed the amplitude modulation of the songs by extracting the song envelope (for details of the procedure see Machens *et al.* 2001; von Helversen *et al.* 2004), and determined several song parameters either by hand for *C. mollis*, F1 hybrid and Backcross males or with a custom-written software (Matthias Hennig, Humboldt Universität zu Berlin, Germany) for males of *C. biguttulus*. Within each song we analyzed phrase duration, pause durations between phrases, period duration, syllable length, pause durations between syllables, syllable to pause ratio, buzz duration, pause duration between buzzes, percentage of buzzes with ticks of a song and the syllable structure of a phrase (terminology after von Helversen 1975a,b, 1997). The percentage of the syllable structure of an individual was calculated as

$$S = \frac{mean\ syllable\ number\ per\ phrase \times mean\ period\ duration}{mean\ phrase\ duration \times 0.01}.$$

The internal phrase/buzz structure of F1 hybrid songs was often highly variable with variation in pulse duration and pulse pauses. In order to reduce the number of false positives I counted only syllables that started and ended with a pause (> 8 ms) and that showed no gaps (> 8 ms) in between. Phrases of pure *C. biguttulus* males start with a ramp and often end with single pulses (von Helversen 1972), this often didn't fit my strict syllable criterion and led to a reduced value for the mean syllable structure (see results, Table 2.2). For

comparisons I followed the idea discussed by von Helversen and von Helversen (1975 a,b), that the phrase in *C. biguttulus* songs is the equivalent song structure to the buzz ('Schwirrlaut') in *C. mollis* calling songs. For each individual at least 8 phrases/buzzes with pauses and at least 30 syllables in the plateau region of the song, from at least 5 songs per individual were analyzed. For further analyses, the average values of a male were used. For the playback experiments with original male songs, I extracted the envelopes of 10 *C. biguttulus* songs, 10 *C. mollis* songs and 12 F1 hybrid songs (6 for each crossing type). After extraction of the envelope the song amplitude was normalized and the envelope was then filled with broad band noise (2–40 kHz). All male songs, which took part in the playback experiment, were recorded from different individuals, which were born and raised in the lab. *Chorthippus biguttulus* and *C. mollis* females were tested with conspecific male songs and songs of F1 hybrid males, whereas Backcross females were tested with songs of *C. biguttulus*, *C. mollis* and F1 hybrid males. Females were exposed 18 times to each male song, which were played back in randomized order (for details about the method of the playback experiments and stimulus generation see Reichert and Ronacher, 2015; Schmidt et al., 2008). Female response probabilities were calculated according to the species of the male songs (*C. biguttulus*, *C. mollis* and F1 hybrid male). All females responded less than three times to the negative control. In total the responses of 26 *C. biguttulus* females, 12 *C. mollis* and 8 Backcross females were analyzed. I used a Kruskal Wallis post hoc test (*kruskal*; R) to test whether female response probabilities differ significantly between songs of the parental species and the F1 hybrids males; with p values adjusted for multiple comparisons using Holm- Šídák procedure and family-wide alpha of 0.05.

In addition, I conducted three experiments to test female preferences to certain F1 hybrid male song characteristics by using artificially generated test stimuli. For the first experiment I varied the proportion of buzzes with ticks and without ticks (0%, 25%, 50%, and 75%). The buzz duration was held constant to 500 ms interrupted by 120 ms pauses and each tick (10 ms) was played 10 ms before buzzes. Buzzes without and with ticks were homogeneously distributed within each song. In the second experiment I varied the proportion of phrases/buzzes with syllables and phrases/buzzes without syllables (5%, 10%, 20%, 30%, 60%, 80%, and 100%). The phrase/buzz duration was held constant to 920 ms interrupted by 120 ms pauses. The syllables (80 ms) were inserted into a phrase/buzz by starting with a 12 ms pause fol-

lowed by the 80 ms syllable and completed by a 12 ms pause. The insertion started in the middle of the phrase/buzzes and extended by increasing syllable numbers to both ends of the phrase/buzz.

For the last experiment in this sequence I held the buzz pause constant to 120 ms and varied the buzz duration from 400–1000 ms in 100 ms steps plus the durations of 1500 ms, 2000 ms, and 2800 ms. In all three experiments the song duration was 8 s. The artificially generated songs of the last three experiments were played back 18 times in randomized order to the females and response frequencies were estimated (for details about the method of the playback experiments and stimulus generation see Reichert and Ronacher, 2015; Schmidt et al., 2008). Females with more than 3 responses to the negative control (3 s of a continuous noise) were discarded as non-selective (*C. mollis* 3/18; *C. biguttulus* 4/17; Backcross 0/9). I analyzed female preference functions by using generalized estimating equations models; one for each comparison (*C. biguttulus* vs *C. mollis*, *C. biguttulus* vs Backcrosses, Backcrosses vs *C. mollis*) for each of the three tests (tick structure, syllable structure, buzz duration). All models were implemented with the *geeglm* function in the *geepack* package (Højsgaard et al. 2006) in R. For details about the model see Reichert and Ronacher (2015), in short: The response variable, modeled as binomial, was the number of female responses to certain test stimulus. The individual female ID was included as a random factor, to account for the repeated measurements within females. As the main effect factors the species/ crossing type and the test stimulus (i.e., the specific song type that was varied) was added to the model. In addition, I added the interaction term for the two main effects to determine if response probability differed between song type and species/crossing type. For the RI calculation I used female response probabilities to original male songs, due to the higher similarity with natural habitat conditions.

Behavioral Isolation of F1 hybrid and Backcross females

To estimate the behavioral isolation strength of F1 hybrid and Backcross females I tested the hypothesis that hybridization affects the response probabilities and the shape of female preference functions. To this aim, I measured three preference functions for different male calling song characteristics (pause duration between buzzes/phrases, pause duration between syllables and syllable duration). For each preference function only a single male calling song characteristic was varied whereas others characteristics were held

constant. Within the first test session the buzz/phrase duration was held constant at 240 ms (2–40 kHz) and the pause duration between to syllable/buzzes was varied (30 ms, 60 ms, 120 ms, 240 ms, 480 ms and 960 ms). This experiment tested for typical *C. mollis* song characteristics and each song was about 8 s long. In the second experiment, which exhibits typical *C. biguttulus* calling song characteristics, the syllable duration of the songs (3 s) was held constant (with 80 ms 5–40 kHz) and the pause duration between syllables was varied (5 ms, 15 ms, 25 ms, 32 ms and 48 ms). The third experiment was designed to test variation of syllable duration of *C. biguttulus* calling song, by varying the syllable duration (30 ms, 60 ms, 80 ms, 100 ms, 120 ms and 240 ms; syllables from noise 5–40 kHz) with constant pause durations of 15 ms. The song characteristics and the range which was tested was based primarily on values used in previous studies of female preferences in these species (von Helversen & von Helversen 1975b, 1994, 1997). In all experiments the songs were broadcast to the female at a sound pressure of 70 dB (see Reichert and Ronacher 2015 for details about testing procedure and stimulus generation). I analyzed female preference functions by using generalized estimating equations models; one for each of the three tests (pause variation with 240 ms syllable duration; pause variation with 80 ms syllable duration, syllable variation with 15 ms pauses) and one for each comparison (parental vs hybrids, parental vs backcrosses and backcrosses vs hybrids). All models were implemented with the *geeglm* function in the *geepack* package (Højsgaard et al. 2006) in R. For details about the model see Reichert and Ronacher (2015). For RI calculation I compared response probabilities of test stimuli with the highest response probabilities of the parental lines (see calling song preference) with the response probabilities of the F1 hybrid and Backcross females to those test stimuli.

Further, I analyzed characteristics of female response songs to determine whether hybridization affected the acoustic signal of females. Female response songs were recorded during previous preference experiments. First, I estimated the mean phrase/buzz duration for each female by averaging all female songs within and across test stimuli. For instance, each preference experiment contained 20 test stimuli, females were exposed to each stimulus 18 times and in case a female responded to 5 test stimuli 16 times with 3 phrases per song each, the mean phrase duration of this female would consist of 240 single measurements. In total the mean buzz/phrase duration of 32 *C. biguttulus*, *25 C. mollis*, 27 F1 hybrid and 8 Backcross females was taken. In

addition, I estimated the mean duration of phrases/buzzes only from the test stimulus which was the most attractive (peak) to the female. Second, I estimated across all response the mean response latency for the first phrase/buzz. In total, I measured response latencies from 32 *C. biguttulus*, 32 *C. mollis*, 32 F1 hybrid and 9 Backcross females. The data of mean duration and response latency were not normally distributed and were analyzed with a Kruskal Wallis post hoc test (*kruskal*; R). I tested the effects of species/crossing type on the phrase/buzz duration and response latency, with p values adjusted for multiple comparisons using Holm- Šídák procedure and family-wide alpha of 0.05.

2.3 Results

Prezygotic barriers

Females of both species showed a significantly lower response probability to a heterospecific calling song model (*C. biguttulus* $\chi^2 1 = 62.1$, $p < 0.001$; *C. mollis* $\chi^2 1 = 14.6$, $p < 0.001$). The difference in the average response to the conspecific test stimulus was remarkable with 55.7% for *C. biguttulus* and 49.2% for *C. mollis* and with 2.4% and 6.9%, respectively, to the heterospecific test stimulus. Thus, the calling song preference has a strong impact on the reproductive isolation between the two species in both crossing directions (RI = 0.92 for *C. biguttulus* x *C. mollis* and RI = 0.75 respectively, Table 2.1). In case a male encountered a female, for example after successful long range courtship behavior, chemical cues contributed as the second barrier in the sequence to reproductive isolation. The relative contribution of male choosiness on chemical cues of females to reproductive isolation differed between the crossing directions (0.49 and 0.23 respectively, Table 2.1). The absolute contribution was with a difference of 3.2% between crossing types rather small (Table 2.1). In the next step I tested the impact of the acoustic signal to the mating success at close range in *C. biguttulus*. In the test trial with intact males 100% of the females were mated after 7 days, whereas 78.6% of females were mated in the test with the muted males. The difference between groups was not significant (Fisher's exact test, $p = 0.22$) and the relative isolation strength of the acoustic signal at close range was 0.12 (Table 2.1). No choice experiments with females of both species tested with heterospecific males revealed strong

prezygotic isolation, even without acoustic signals. None of the females were mated independent of whether females were tested with intact or muted males. Thus, the RI was 1 for both crossing directions. In order to be able to estimate the impact of the following barriers, I decided to exclude this result from further calculation of the absolute contribution of a barrier to the total isolation.

Table 2.1 Reproductive isolation barriers between *C. biguttulus* and *C. mollis*.

The components of reproductive isolation (RI), the cumulative strength and the absolute contribution of reproductive barriers are given relative to the crossing direction of the pure species. The result of the last prezygotic barrier (grey box) was excluded from further calculation to assess the strength of reproductive isolation of the following barriers. All values were calculated based on the equation 4A and 4E described in Sobel and Chen (2014). The mating success of acoustic signal was not tested (NT) in *C. mollis*.

	C. biguttulus[1] x *C. mollis*[2]			*C. mollis*[1] x *C. biguttulus*[2]		
Reproductive isolation barriers	RI value	cumulative strength	absolute contribution	RI value	cumulative strength	absolute contribution
prezygotic barriers						
calling song preference	0.917	0.9174	0.917	0.7540	0.7540	0.754
chemical cues short range (male choosiness)	0.486	0.9706	0.053	0.2308	0.8388	0.085
acoustic signal short range (mating success)	0.12	0.9768	0.006	NT	NT	NT
{mating success no choice experiment (chemical cues)	*1*	*1.0000*	*0.023*	*1*	*1.0000*	*0.1612}*
postzygotic barriers						
hatching success of fertilized eggs	0.292	0.9872	0.010	0.2416	0.8984	0.060
survival rate of larvae	0.096	0.9894	0.002	0.1037	0.9167	0.018
functional development of wing & hind leg morphology	0.099	0.9913	0.002	0.0841	0.9291	0.013
courtship motivation of females	0.607	0.9979	0.007	0.6211	0.9830	0.054
behavioral isolation of F1 hybrid males (acoustic)	0.666	0.9996	0.002	0.6960	0.9969	0.014
behavioral isolation of F1 hybrid females (acoustic)	0.727	0.9999	3.6E-04	0.7265	0.9995	0.003
hatching success of fertilized backcross eggs	0.237	1.0000	2.6E-05	0.1852	0.9997	2.0E-04
survival rate of backcrosses larvae	0.231	1.0000	1.6E-05	0.1214	0.9997	7.3E-05
functional development of wing & hind leg morphology BC	0.115	1.0000	5.4E-06	0.1006	0.9998	4.8E-05
courtship motivation of backcross females	0.017	1.0000	6.8E-07	-1.9E-05	0.9998	-8.3E-09
behavioral isolation of Backcross females	0.06	1.0000	2.3E-06	0.4777	0.9999	1.4E-04
Total isolation		**1.0000**			**0.9999**	

1 conspecific species

2 heterospecific species

Intrinsic postzygotic isolation

Hatching success

The hatching success of F1 hybrid larvae (49.4%) and Backcross larvae (55.6%) was significantly reduced compared to the hatching success of *C. biguttulus* larvae (90.1%) and *C. mollis* larvae (80.9%). The generalized linear model on hatching success revealed a significant difference between crossing types ($p < 0.001$). Post hoc tests showed a significantly lower hatching success of both F1 hybrid crossing directions compared to both parental species and Backcrosses (Table A.1). The hatching success of Backcrosses was also significantly lower compared to *C. biguttulus* and *C. mollis*. The post hoc test showed no significant differences in hatching success for the comparison *C. biguttulus* and *C. mollis* and for the comparison between crossing direction of F1 hybrids (Table A.1). The relative contribution of F1 hybrid hatching success as intrinsic postzygotic barrier was similar between the parental species *C. biguttulus* (0.29) and *C. mollis* (0.24) (Table 2.1). For backcrosses the relative contribution of hatching success was with 0.24 for *C. biguttulus* and 0.19 for *C. mollis* slightly smaller compared with F1 hybrids (Table 2.1). Thus, the RI value in backcross and F1 hybrid comparisons is negative (-0.06, Table A.2).

Survival rate of larvae

The F1 generations differed significantly in the survival rates between the crossing types (χ^2 4 = 37.67, $p < 0.001$). The offspring of *C. biguttulus* showed with 81.51% the highest larvae survival rate. The survival rate of *C. mollis*, backcrosses, BIMO hybrids and MOBI hybrids was lower with 62.04%, 50.82%, 51.43% and 50.38%, respectively. Post hoc test showed that only the survival rate of *C. biguttulus* is significantly different to that of all other groups (Table A.1).

Functional development of wing and hind leg morphology

Twelve percent of F1 hybrids and 20% of Backcrosses showed a trend for higher disorders in functional development of compartments which produce the acoustic signals, although the difference to *C. biguttulus* (0%) and *C. mollis* (2.9%) was not significant. The relative isolation strength was 0.099 for F1 hybrids to *C. biguttulus* and 0.084 to *C. mollis*. For the comparisons of Backcrosses to *C. biguttulus* the relative isolation strength was 0.115 and to *C. mollis* 0.101. The RI value of Backcrosses to F1 hybrid comparison was with 0.017 very small (Table A.2).

Internal sexual organs

I found no evidence for differences between species and crossing types in ovariole numbers of females (Kruskal Wallis χ^2 2 = 0.3, p = 0.86). The mean (± sd) ovariole number for *C. biguttulus*, *C. mollis* and F1 hybrids was 8.4 ± 1.6, 8.4 ± 1.3, 8.7 ± 1.3, respectively. To test the impact on hybridization on the internal sexual organs in males, I first examined whether femur length can be used for body size correction. The femur length and testis weight were positively correlated (Pearson correlation 0.45; p = 0.015), indicating that testis weight depends on body size. An ANCOVA test revealed no interaction between species/crossing type and femur length, demonstrating that femur length correlates with testis weight in all groups.

The interaction term was removed from the model and the new model ($p < 0.01$) showed significant differences between crossing types and testis weight (Figure 2.2). The post hoc test revealed that only the crossing type *C. mollis* female with a *C. biguttulus* male (MOBI) is significantly different from the testis weight of *C. biguttulus* males (p = 0.004; Figure 2.2). The lower testis weight is in line with the low fertilization success of F1 hybrid males (see 'Hybridization experiments' in the methods)

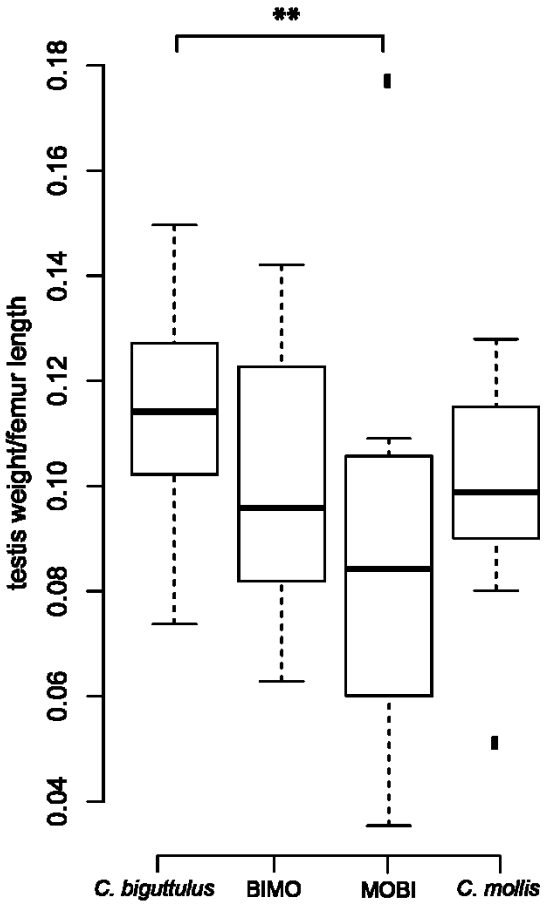

Figure 2.2 The effect of hybridization on testis weight.
The ratio of testis weight and femur length from 13 *C. biguttulus*, 13 *C. mollis* males and offspring males of reciprocal crossing (11 BIMO and 11 MOBI males). Testis weight differ significantly between *C. biguttulus* and MOBI males (post hoc Tukey test, p = 0.004).

Extrinsic postzygotic isolation

Courtship motivation of females

I estimated the courtship motivation level of females to examine the behavioral isolation strength. The motivation level between test groups differed significantly (Kruskal Wallis χ^2 3 = 38.61, $p < 0.001$) driven by a strong decrease in response motivation of F1 hybrids (Posthoc Kruskal Nemenyi for comparisons with parental species both $p < 0.001$ and for F1 hybrids and backcross comparison $p < 0.01$; Figure 2.3). The strong effect of hybridization in courtship motivation resulted in high RI values (0.61 to *C. biguttulus* and 0.62 to *C. mollis;* Table 2.1). The courtship motivation of Backcross females (52%) was equally high as the courtship motivation of the parental lines (*C. biguttulus* 53.8%; *C. mollis* 52%) thus motivation effects on reproductive isolation of Backcrosses were very weak (Table 2.1). The higher courtship motivation of Backcrosses (52%) compared to F1 hybrids (11.8%) resulted in a negative RI value (Appendix Table A.1).

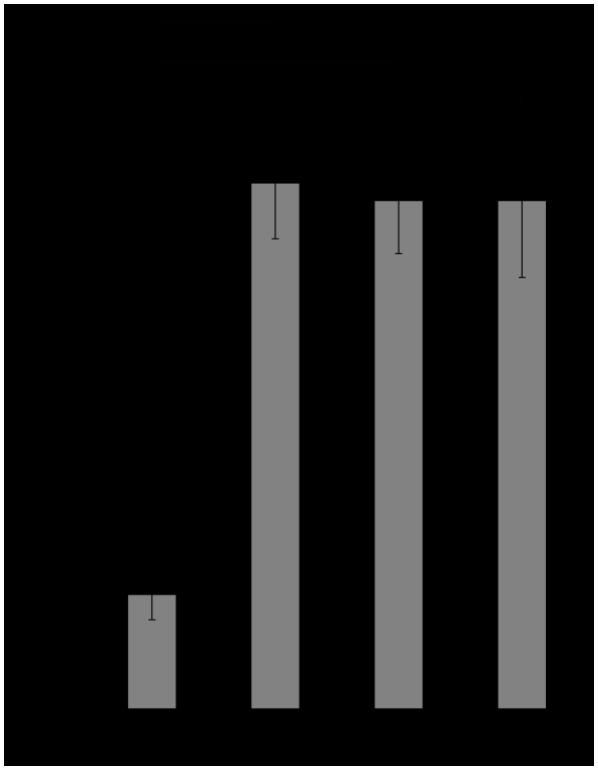

Figure 2.3 Effects of hybridization on female courtship motivation.
Significant levels between species groups are indicate by stars (** $p < 0.01$, *** $p < 0.001$). The sample size for each group is given in brackets.

Behavioral isolation of F1 hybrids males

The phenotypical characteristics of hybrid songs were intermediate for almost all parameters which were analyzed (Table 2.2). The occurrence of longer buzzes in F1 hybrid males supports the homology of the phrase and the buzz (von Helversen & von Helversen 1975a). One exception for non-intermediate song traits of hybrids was the syllable and pause duration. Whenever a hybrid male produced syllables, the duration of the syllables and the pauses was close to the syllable durations and pause durations of *C. biguttulus* males. The calling songs of the Backcrosses were very similar to those of *C. biguttulus.*

Table 2.2 Song characteristics of *C. biguttulus*, *C. mollis*, F1 hybrids & Backcross males.

Song characteristics with dash indicate that this characteristic was not present in the species group. Values are the mean of all individuals' means, the standard deviation (sd) and the coefficient of variance (CV) in percent.

species group		phrase/buzz duration [ms]			phrase/buzz pause [ms]			syllable [ms]			pause [ms]			syllable to pause ratio		syllable struct. of phrases [%]		tick struct. [%]	
	N	mean	sd	CV	mean	sd	CV	mean	sd	CV	mean	sd	CV	mean	sd	mean	sd	mean	sd
C .biguttulus	21	2889	372	12.9	2645	284	10.8	81.8	13.7	16.8	20.1	3.2	16.1	4.1	1.0	78.5	8.5	-	-
C. mollis	16	414	59	14.2	123	21	16.7	-	-	-	-	-	-	-	-	-	-	94.0	9.7
BIMO	13	923	156	16.9	352	320	91.1	69.7	10.5	15.0	12.7	1.6	12.8	5.5	0.8	5.2	5.0	29.4	25.6
MOBI	18	807	139	17.2	287	93	32.5	84.1	23.8	28.4	13.7	2.5	18.3	6.1	1.8	9.9	6.4	35.1	23.1
Backcrosses	7	2219	522	23.5	1677	505	30.1	81.0	15.6	19.2	16.1	2.5	15.5	5.0	1.0	69.7	8.1	-	-

Both parental species answered original male hybrid songs less frequently ($p < 0.001$, Figure 2.4). The response proportion of backcross females differed significantly between responses to *C. biguttulus* males songs and responses to *C. mollis* or F1 hybrid male songs ($p < 0.001$), but not between responses to *C mollis* males songs and F1 hybrid males songs ($p = 1$, dark bars in Figure 2.4). Pairwise comparison between *C. biguttulus* and Backcross females in response proportion showed not differences ($p = 0.662$). According to these results, the calculated RI values to *C. biguttulus* (0.67) and to *C. mollis* (0.7) were relatively high (Table 2.1). Additional playback experiments that tested hybrid song characteristics based on the results of Table 2.2 confirmed the low attractiveness of hybrid songs (Figure 2.5 A-C).

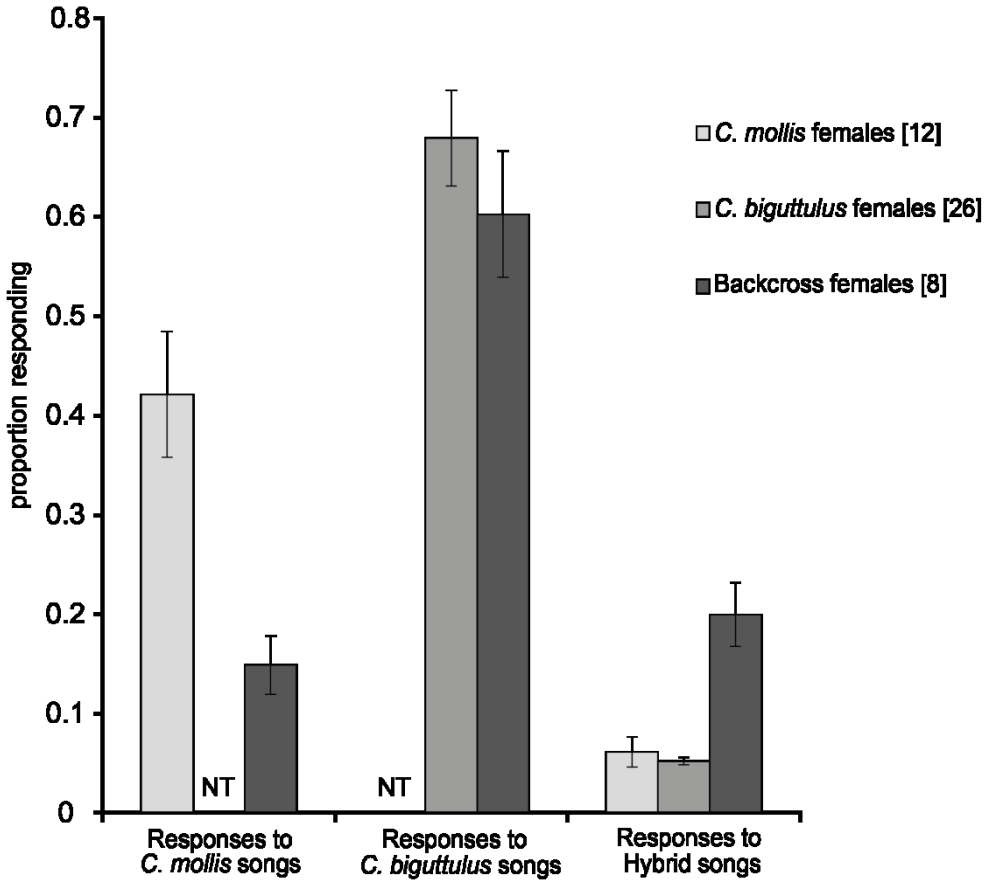

Figure 2.4 Playback experiments with original male songs.
Bar plot displays the mean ± SE proportion of times female responded to 10 *C. biguttulus*, 10 *C. mollis* and 12 F1 hybrid songs. The number of tested females is given in brackets. *Chorthippus biguttulus* and *C. mollis* females were not tested (NT) with heterospecific male songs.

In the first experiment, the response frequencies of *C. mollis*, *C. biguttulus* and Backcross females were independent of the tick structure (Figure 2.5 A). *Chorthippus mollis* showed with an average response of 30% the highest response probability compared to *C. biguttulus* and Backcross females, indicating that these song features: buzz/phrase duration of 500 ms interrupted by very short pauses of 120 ms were too short to match the preference of *C. biguttulus* and Backcross females. The second experiment demonstrated, in addition, that syllable structure of a male song was essential to elicit high response frequencies in *C. biguttulus* and Backcross females (Figure 2.5 B). *Chorthippus biguttulus* and Backcross females showed a significant increase in response frequency to test pattern with a syllable structure of ≥ 60%. The response frequency of *C. mollis* females was significantly lower compared to *C. biguttulus* and Backcross females and independent of the syllable structure of the test pattern (Figure 2.5 B). The attractiveness of long buzzes produced by F1 hybrid males was tested in the last experiment (Figure 2.5 C). The response curve of *C. mollis* continuously decreased starting from high response probabilities for short buzz durations (400 ms, 500 ms and 600 ms). In contrast, the response frequency of *C. biguttulus* and Backcross females was low and showed no preference for any test pattern (Figure 2.5 C). Statistics of comparisons of species, song variant and the two way interactions of these two variables are summarized in Table A.4 for all three experiments (tick structure, syllable structure and buzz duration).

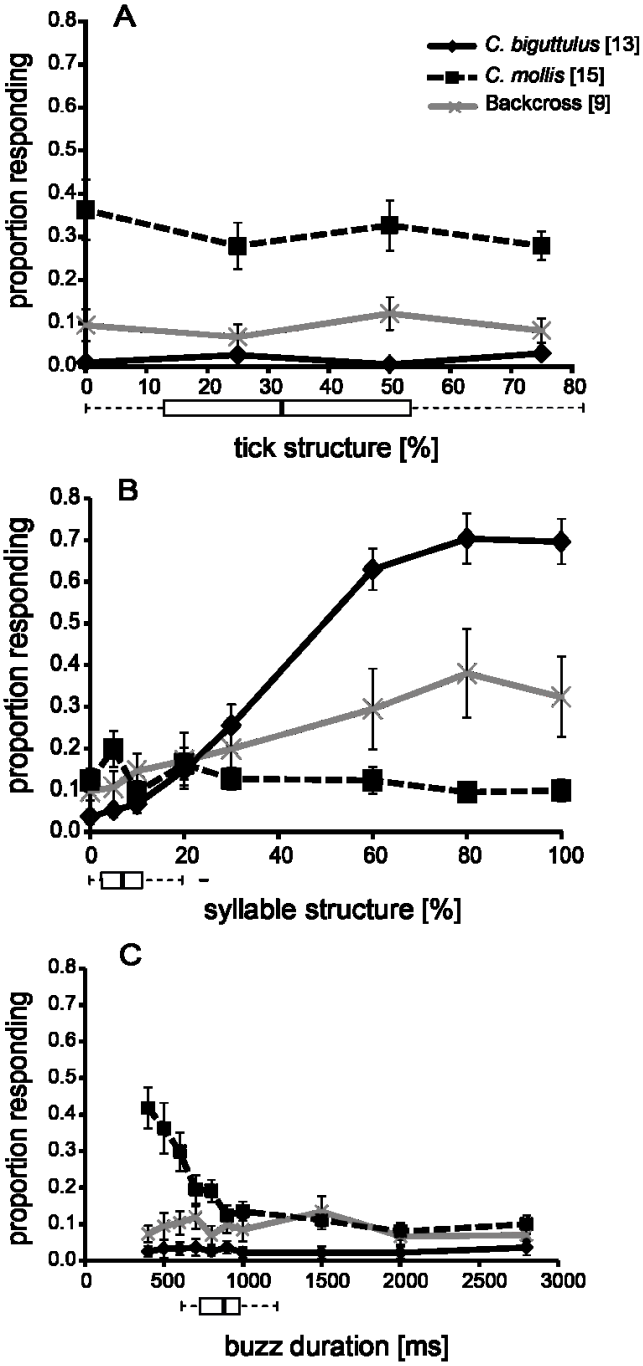

Figure 2.5 Female preference functions for each of the three male songs characteristic. Artificial song models varied **A**) in tick structure, **B**) in syllable structure and **C**) in buzz duration. Preference functions are averaged across all females that were tested with these stimuli. Each data point represents the mean ± se proportion of times that females responded to a given stimulus. Legend illustrates the corresponding species group and sample size. The horizontal box plots below the x axis represent the male songs trait of F1 hybrid males (Table 2.2) for each of the three tested features.

Behavioral isolation females

Hybridization strongly affected the female preferences for male calling song characteristics (Figure 2.6). The preference functions of hybrid females were affected in peak preference (low or no peaks), responsiveness (reduced responses) and preference strength (flat shape) (Figure 2.6). In contrast, the preference curves of Backcross females were intermediate with a tendency towards higher similarity to *C. biguttulus* preference curves. *Chorthippus mollis* females showed a clear preference for 240 ms pauses between two buzzes with a constant duration of 240 ms (Figure 2.6 A). This response profile was significantly different from all other groups (for all comparisons $p < 0.001$). All other groups were not significantly different from each other (Table A.5). The response frequencies of *C. biguttulus* females showed a peak for pauses with 30 ms duration, followed by a continuous decrease of responses to longer pause durations (Figure 2.6 A). Hybrid females of both crossing directions showed a slightly higher response frequency to a pause duration of 60 ms, followed by a decrease to longer pauses. Backcross females showed no peak to any pause duration, although the overall response frequency was slightly higher compared to *C. biguttulus* and F1 hybrids.

In the second and third experiment *C. biguttulus* and Backcross females showed similar preference peaks (in 2.6 B both for 80 ms syllable duration, in 2.6 C both for 15 ms pause duration) but preference curves differed significantly (variation syllable duration: $\chi^2 1 = 7.15$, $p = 0.008$; variation pause duration between syllables: $\chi^2 1 = 5.42$, $p = 0.02$) and also to all other groups (Figure 2.6 B, C, Table A.5). Females of *C. mollis* and females of the crossing type MOBI showed no preference in both experiments (Figure 2.6 B, C) for any of the tested syllable durations or pause duration and were not significant different from each other (Table A.5). Hybrids of the crossing type BIMO responded with slightly higher frequency to syllable pauses of 15 ms (Figure 6 C) compared to the crossing type MOBI, although the overall response frequencies was not significantly different between BIMO and MOBI (variation pause duration between syllables: $\chi^2 1 = 0.53$, $p = 0.466$).

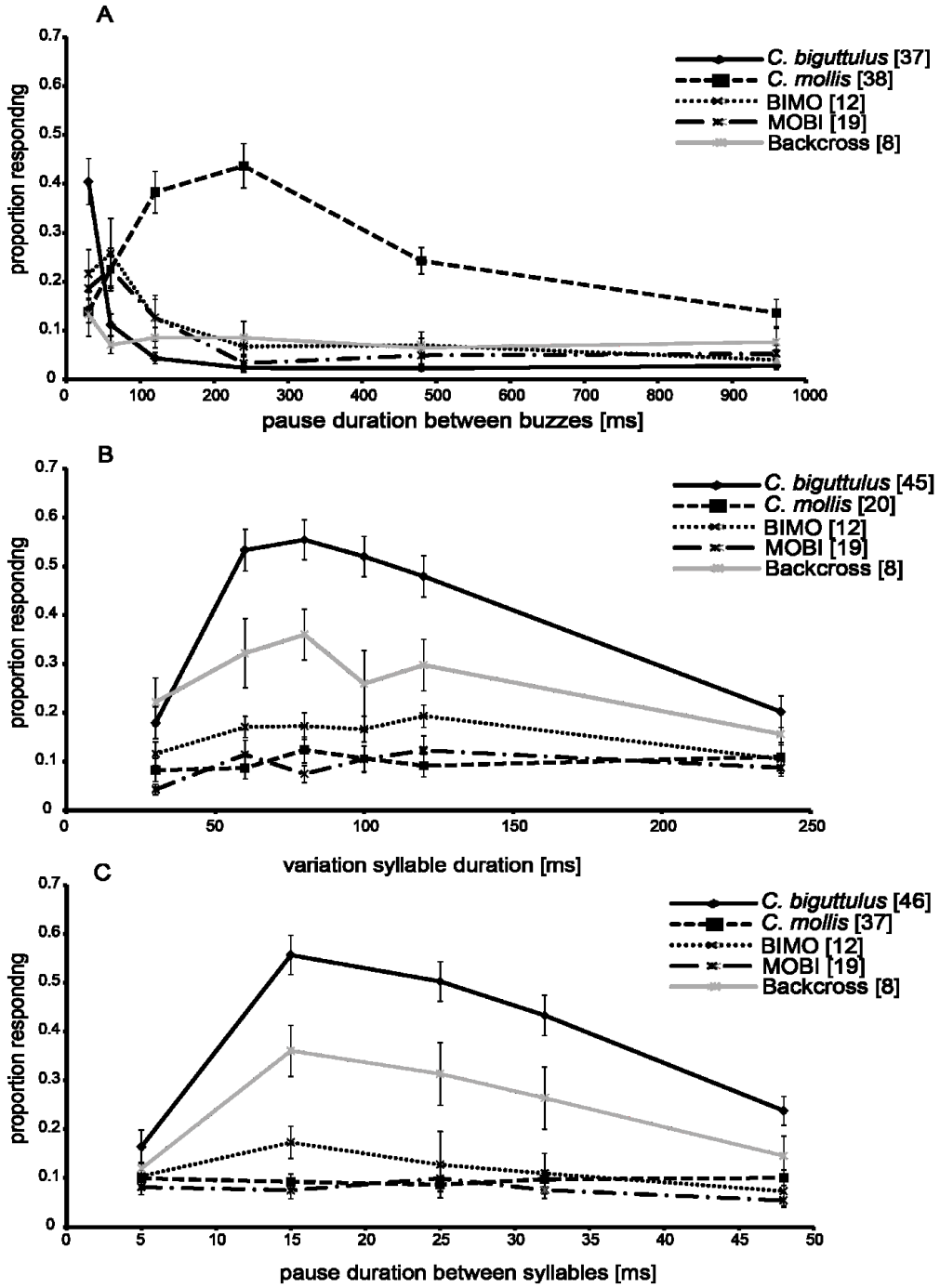

Figure 2.6 Preference functions of parental species, F1hybrids & Backcrosses females.
In this experiment, three trial types were used that tested for: **A**) pause duration between buzzes, **B**) syllable duration and **C**) pause duration between syllables. Preference functions are averaged across all females that were tested with these stimuli. Each data point represents the mean ± SE proportion of times that females responded to a given stimulus. Legend illustrates the corresponding species group and sample size.

The strong impact of hybridization on female preference functions resulted in high RI values for F1 hybrids (72.7%) for the crossing with *C. biguttulus* as conspecific species and *C. mollis* as the heterospecific species, but also for the reciprocal crossing direction with RI values of 72.7% for F1 hybrids and 47.8% for Backcrosses (first and fourths column in Table 2.1). The response frequencies of Backcross females to the peak preference of the parental species were for all tests higher than the response frequencies of the F1 hybrids which resulted in negative RI values (Table A.2).

Analysis of female response songs revealed intermediate song traits of F1 hybrid females similar to male song characteristics. The phrase/buzz duration differed not significantly between *C. biguttulus* and Backcross females. The buzz/phrase duration was significantly reduced in F1 hybrids and in *C. mollis* females compared to Backcrosses and *C. biguttulus* females, but F1 hybrid males produced on average longer buzzes/phrases than *C. mollis* females (Figure A.1). The comparison between F1 hybrid and *C. mollis* demonstrated also significant differences (Figure A.1). Pairwise comparisons showed no differences in the mean duration of phrase/buzz and the response duration to the peak phrase/buzz. Thus, results are plotted in Figure A.1 only for the mean phrase/buzz duration.

The average response latency to the first phrase/buzz was significantly longer compared to the response latency of the most attractive test stimulus (peak). The peak response latency was in all groups smaller compared to the average response latency. The average response latencies of F1 hybrids and Backcrosses were not intermediate. The latency in F1 hybrids was similar to *C. mollis* and Backcrosses showed high similarity to *C. biguttulus* (Figure A.2). Only, the response peak latency was intermediate to the parental species for F1 hybrids (Figure A.2), whereas the latencies of Backcrosses and *C. biguttulus* females were not significantly different.

Strength of reproductive isolation barriers

Both crossing directions are strongly reproductively isolated from one another. The isolation strength of the crossing direction *C. biguttulus* x *C. mollis* was complete after the F1 generation (hatching success of fertilized backcross eggs). For the reciprocal crossing type total isolation was 0.999906972078796 (Table 2.1). The experiment on prezygotic isolation barriers revealed high reproductive isolation based on long range courtship behavior (0.92 & 0.75, Table 2.1). However, the acoustic signal had only a minor impact on the mat-

ing success at close range in *C. biguttulus*. The behavioral data extracted from chapter 4 showed that male choosiness to female CHCs also contributes to reproductive isolation. No-choice experiments revealed complete reproductive isolation of *C. biguttulus* and *C. mollis* in both directions (RI = 1, Table 2.1). In order to estimate the absolute strength of the following postzygotic isolation barriers I excluded this result from the following RI calculations. However, even without taking the last result into account the cumulative strength of the previous prezygotic isolation barriers reached 0.9768 & 0.8388, respectively for the two crossing directions (Table 2.1).

The high RI values of extrinsic postzygotic isolation barriers (behavioral isolation and courtship motivation of females) contributed strongly to the total isolation between species. Hybrids were also strongly affected by intrinsic postzygotic barriers (hatching success, testis weight, and survival rate of larvae). The reduced hatching success of hybrids (0.29 to *C. biguttulus* & 0.24 to *C. mollis*) relative to the parental species was the strongest intrinsic postzygotic barrier. However, the effects of hybridization on testis size were not included in Table 2.1, since data about how a smaller testis might affect reproductive success of males are lacking. The cumulative isolation strength of postzygotic barriers alone (0.9985 & 0.9988) was almost as high as in combination with prezygotic isolation barriers (1 & 0.9999), respectively, for crossing directions (see Table 2.1, A.3).

2.4 Discussion

I identified multiple pre- and postzygotic reproductive isolation barriers which differed in strength and for crossing direction. The RI estimates in Table 2.1 convincingly show that reproductive isolation between species is either complete or almost complete for both crossing types. Interestingly, the strengths of pre- and postzygotic barriers were similar when I compared these two separately (Table 2.1, A.4). This indicates that the speciation continuum is relatively far advanced and ongoing gene flow very unlikely. The high RI values of prezygotic barriers suggest an important role for sexual selection in the maintenance of species isolation. Based on the estimates of the strengths of pre- and postzygotic barriers, potential mechanisms for the evolution of reproductive isolation barriers and their role during speciation will be discussed.

Prezygotic barriers

The first barrier in the sequence of reproductive barriers was female preference on acoustic male signals. This prezygotic barrier acts first in (long range) mate attraction and females showed strong preferences for the conspecific male song model which resulted in high RI values (0.92 and 0.75, Table 2.1). This barrier appears to be asymmetric, as the RI values are different for crossing directions. However, the reduced RI value for *C. mollis* x *C. biguttulus* crosses was not only due to the higher acceptance of *C. mollis* females for the heterospecific male song model, but also due to the lower response frequency to the conspecific male song model. Both effects in combination caused the reduced strength of this barrier for *C. mollis* x *C. biguttulus* crossing relative to the reciprocal cross. Asymmetric strength of reproductive isolation barriers is found in many species (Coyne & Orr 2004). The Kaneshiro effect is one possible explanation for this observation, proposing that the derived species is less discriminant on sexual signals as the ancestral species (Kaneshiro 1976, 1980). However, the asymmetry between the grasshopper species was very low and the conditions under which the Kaneshiro effect is predicted to occur is controversial (Ehrman & Wasserman 1987). Therefore, further research is needed to confirm that the asymmetry between the species is stable and significant and to rule out other factors that might have driven this pattern.

In general, the female preferences results are in line with previous studies that female preferences do not overlap between the two species and that acoustic signals are considered as a strong component of reproductive isolation between these species, but also for other species of the subfamily Gomphocerinae (Mayer *et al.*, 2010; von Helversen, 1997; von Helversen & von Helversen, 1975a,b, 1994). However, multiple studies demonstrated that reproductive behavior of females, in many grasshopper species, is characterized by phases of passive receptiveness, which means that many females frequently mate without prior response stridulations (Riede 1983; Butlin & Hewitt 1986; Wirmer *et al.* 2010). Furthermore, Butlin and Hewitt (1986) argued that response stridulation of females is more a sign of 'sex starved' females and that most matings under natural conditions result from chance contacts (Kriegbaum 1988). These observations indicate that additional communication cues are important to prevent heterospecific matings and

might therefore be involved in reproductive isolation (Butlin *et al.* 1985; Ritchie 1990; Kriegbaum & von Helversen 1992; Butlin 1998).

In order to estimate the reproductive isolation strength of short range cues I used the data from two behavioral experiments. The first barrier is male choosiness to court conspecific or heterospecific females based on CHC cues. The isolation strength of this barrier was again asymmetric between species pairing, which was caused by higher calling frequency of *C. mollis* males to heterospecific female CHC. However, even the calculated RI value for *C. mollis* x *C. biguttulus* crosses was only half compared to the reciprocal cross (0.49 & 0.23), the difference in absolute contribution between species pairing was with 3.2% small (Δ0.053 - 0.085, Table 2.1). Furthermore, the no choice experiments demonstrated that non-acoustic signals, most likely CHC signals, are sufficient and essential for mating decisions at short range. In contrast, the presence of a conspecific acoustic signal was not essential for mating success (tested for *C. biguttulus*). Singing males in these tests led to a higher proportion of mated females, but this increase was not significant. The total isolation between species pairing was almost complete after estimating the isolation strength of prezygotic barriers, which clearly shows the importance of those prezygotic barriers in the maintenance of these species.

Nevertheless, this data should be handled with care as lab artifacts might have cause high RI values. First, if we follow the idea of Butlin and Hewitt (1986), that the response stridulation of females under natural condition is less relevant for mate location and mating success, it would cause an overestimation of the reproductive isolation strength. However, the impact of this barrier on reproductive isolation might increase in low population densities when this behavior becomes more important (Butlin & Hewitt 1986). Second, the high RI values in no choice experiments when females were confronted only with muted heterospecific males would be potentially reduced in a more natural situation. For instance, the error rate in females to avoid heterospecific matings might be higher, when females were exposed to conspecific and heterospecific male songs which would lower the RI value. Nonetheless, female and males were in hybridization experiments very resistant to mate with heterospecific partners even if they were stimulated only with conspecific songs. This fact reduces the likelihood that the high RI values in no choice experiments are due to the experimental design.

This raises the question about the role of the acoustic signal in the maintenance of species isolation. The no choice and hybridization experi-

ments showed that the acoustic signal was not essential for mate stimulation or for preventing hybridization. However, female preference functions on acoustic male signals are species–specific and narrowly shaped, indicating strong selection forces on female preference and an important role in mating success. Field experiments in *C. biguttulus* demonstrated that a higher proportion of females were mated earlier in their lifetime when the populations consisted of singing males compared to a population with muted males (Kriegbaum & von Helversen 1992). Additional experiments showed that frequency for a singing male and a muted male to meet a female differed not significantly (Kriegbaum 1988). This supports the hypothesis that the acoustic signal is less important for mate localization and identification than for mate stimulation (Butlin & Hewitt 1986). Summarizing previous findings and the results of this study it seems that the role of acoustic communication in reproductive isolation is less important and that chemical cues are dominant in the maintenance of species isolation.

Intrinsic postzygotic barriers

The authors of previous hybridization studies claimed that no intrinsic postzygotic barriers were present in the same species pair (von Helversen & von Helversen, 1975a,b) or in a closely related species (Perdeck 1958; Gottsberger & Mayer 2007). However, these barriers were not explicitly tested or quantified. In contrast, my results revealed strong intrinsic postzygotic isolation. The first intrinsic postzygotic barrier was the reduced hatching success of F1 hybrids, which was caused by developmental disorders of embryos (fertilization rate of unhatched eggs = 89.8%). The hatching success of Backcrosses was slightly higher compared to F1 hybrids, but in contrast to F1 hybrids were many Backcross eggs not fertilized (fertilization rate of unhatched eggs = 26.2%). These results indicate that the low hatching success of F1 hybrids and Backcrosses is based on two different reasons. In F1 hybrids developmental disorders of embryos is suggested as the major reason, whereas the low hatching success of Backcrosses seemed to be caused by the low fertility of F1 hybrid males. All F1 hybrid males were incapable to fertilize eggs. The testicular weights of F1 hybrid males were reduced as compared to parental males, but with differences between crossing directions (Figure 2.2). Hence, this is another example of Haldane's rule, since the reproductive organs of females were not affected and the males (X0) are the heterogametic sex in *C. biguttulus* and *C. mollis*. Hewitt et al. (1987a) reported

similar results for interspecific crosses between two subspecies of *C. parallelus*. In males of the grasshopper species *Myrmeleotettix maculatus*, sperm dysfunctionality was correlated with B chromosome frequency (Hewitt *et al.* 1987b). B chromosomes can modify meiotic processes and are taxonomically widespread across a wide range of species with high intra- and interspecific frequency variation (Jones & Rees 1982). One possible explanation for the spermatogenic dysfunction observed here might be due to a disturbance of the X chromosome during mitosis and meiosis processes, like in *Drosophila* (Hewitt 1979; Lindsley & Tokuyasu, K. 1980; Hewitt *et al.* 1987a). Applied to my results, differences in crossing direction may have occurred due to species differences in B chromosome frequency in combination with a sex bias in transmitting rate of B chromosomes, like in *M. maculatus* (high transmitting rate in females, low transmitting rate in males)(Hewitt 1973). In addition, hybridization disturbs the balance of autosomal genes which control the X chromosome in males. It is possible that this unbalance was in MOBI males higher due to additive effects of B chromosomes. Definitely, the disorders in embryo development together with the virtually sterility of F1 hybrid males demonstrate strong genetic incompatibilities of both reciprocal crosses.

In contrast, larvae survival seemed to be not strongly affected by hybridization (Table 2.1). Remarkably, the survival rate of the parental lines was surprisingly low, especially in *C. mollis*. It is possible that the grasshopper were infested by parasites. The survival rate of larvae of the parental species was in following years higher (> 80%), which would then result in higher RI values.

I quantified the functional development of the external sound producing organs of the grasshoppers as the last intrinsic postzygotic barrier. F1 hybrid and Backcross individuals showed a tendency to have a higher deformation rates in these organs, but the difference to the parental species was not significant. The RI values of the larvae survival and the functional development of sound producing organs indicate that genetic incompatibilities after embryogenesis are much weaker, but still may affect important developmental processes in the larvae.

Extrinsic postzygotic barriers

The reduced courtship motivation of F1 hybrid females is predicted to reduce gene flow between the parental species. One explanation is that this is mainly caused by the intermediate preference functions of F1 hybrid females.

However, previous experiments suggested that preference functions of hybrid females are not intermediate (von Helversen & von Helversen, 1975b). Alternatively, genetic incompatibilities might lead to a fitness loss of female with consequences in courtship behavior (Coyne & Orr 2004). A F_{st} outlier analysis between *C. mollis* and *C. biguttulus* revealed fixed differences in Calmodulin, a protein which regulates the production of nitric oxide (NO) in nervous tissues (Berdan *et al.* 2015). Weinrich et al. (2008) demonstrated that sound production in *C. biguttulus* females was suppressed by NO injection. Thus, the NO synthesis pathway in F1 hybrid females might be disturbed by incompatible Calmodulin proteins which then cause behavioral anomalies during courtship and mating behavior. Therefore, it is likely that the reduced courtship motivation of F1 hybrid females is due to genetic incompatibilities, but affects the interaction of the hybrid females with their environment (other individuals) therefore this barrier is considered as an extrinsic postzygotic barrier.

Behavioral isolation of F1 hybrid males

Sexual selection against hybrids is mainly caused by the fact that hybrids have intermediate mating traits compared to parental species which makes them unattractive as mates for parental species (Stratton & Uetz 1986; Seehausen *et al.* 1999; Naisbit *et al.* 2001). F1 hybrid males showed intermediate song traits for phrase/buzz duration and pause duration between phrases/buzzes. The same traits were also intermediate in Backcross males, but with a higher similarity to *C. biguttulus* songs. Playback experiments using parental and Backcross females confirmed the non-attractiveness of phrase/buzz durations produced by F1 hybrid males and as a consequence the sexual selection against them (Figure 2.5 C). The phrase/buzzes in this test trial lacked a syllable structure which most likely caused the low response frequency of *C. biguttulus* females (discussed in the paragraph below)(von Helversen 1972). Female preference tests have shown that *C. mollis* females are reluctant to accept buzz durations > 500 ms (Figure 2.5 C), whereas *C. biguttulus* females prefer longer phrase durations (von Helversen 1972; von Helversen & von Helversen 1994). The range of *C. mollis* female preferences for pause durations between phrases or buzzes was much broader (Figure 2.6 A) and thus, expected to play only a minor role in sexual selection (see vatiation in Table 2.2, von Helversen & von Helversen, 1997; von Helversen & von Helversen, 1975 b).

The majority of F1 hybrid male songs had no internal syllable structure. Syllables occurred only in less than 10 percent of the phrases/buzzes of F1 hybrid male songs, but if syllables were present the duration was similar to those of *C. biguttulus* songs (Table 2.2). Although, the syllable to pause ratios were increased in F1 hybrid and Backcross males, but they were still in the range of female preferences (von Helversen 1972; von Helversen & von Helversen 1994). Playback experiments confirmed the prediction that F1 hybrid male songs are unattractive to *C. biguttulus* females due to the lacking internal syllable structure of phrases (Figure 2.5 B). The proportion of responses in *C. biguttulus* females was high for male songs models with a syllable structure of 60% and higher which perfectly matches the measured mean syllable structure of *C. biguttulus* male songs (78.5%, Table 2.2).

In contrast to the syllable structure, the reduced number of ticks before buzzes in F1 hybrid male songs didn't affect the response behavior of females. Ticks are characteristic for *C. mollis* songs, but surprisingly this seems to be not relevant for attraction, at least under lab conditions. It is likely that this tick structure becomes more important under natural conditions where noise may interfere with male songs.

The behavioral isolation of F1 hybrid male songs for *C. biguttulus*, *C. mollis* and Backcross females was primarily based on the combination of long buzzes with no syllable structure (Table 2.1, Figure 2.5) (von Helversen & von Helversen, 1975 a,b). The phrase/buzz duration is controlled by command fibres and the supraesophageal ganglion (protocerebrum) in the brain of grasshoppers, which activates and deactivates the pattern generators in the metathoracic ganglion (Elsner & Huber, 1969; Hedwig & Heinrich, 1997; Hedwig, 1994; Heinrich *et al.*, 2001; Lins & Elsner, 1995). A study on the neuronal and genetic basis of courtship song production in *Drosophila melanogaster* showed that when the protein mosein was misexpressed in the lateral protocerebrum the interpulse interval and cycle per pulse of a song were significantly increased compared to the wild type song (see fly line 003 in Moran and Kyriacou, 2009). This protein was an F_{st} outlier in *C. mollis* and *C. biguttulus* comparison, demonstrating that the gene is under selection and contains nucleotide substitutions (Berdan *et al.* 2015). Thus, the mosein gene is a candidate gene to be involved in generation of the intermediate phrase/buzz durations of F1 hybrid male songs.

The internal structure of a phrase/buzz is predicted to be controlled by a hemisegmental pattern generators in the metathoracic ganglion

(Ronacher 1989, 1991). Dissection experiments in the metathoracic ganglion of *C. biguttulus* individuals revealed, beside several other effects, that hemisection led to longer syllables and, correspondingly, to an increase of the syllable pause ratio (Ronacher 1989). This suggests that the syllable pause ratio is affected when the functionality of inter–ganglion connection is disturbed by dissection or by potential genetic incompatibilities due to hybridization. Von Helversen and von Helversen (1975a) hypothesized that F1 hybrids express the neuronal pattern generator of both parental species which then leads to the occurrence of hybrid songs with characteristics of both parental species. By following this hypothesis the phenotype distribution in Backcrosses would be 50% *C. biguttulus*-like individuals and 50% F1 hybrid–like individuals, when assuming that pure species are homozygous for this trait and that crossing direction has no effect. Considering the low sample size of recorded Backcross males [7] conclusions are speculative, but I found no similarities in any Backcross song with a F1 hybrid song (data not shown). An alternative explanation is, in contrast to the von Helversen and von Helversen (1975) hypothesis that a heterozygous genotype leads to an intermediate pattern generator, instead of two independent neuronal circuits. For instance, the trait for tick structure of *C. mollis* songs is not present in *C. biguttulus* songs, which means that the question is not, whether the tick itself is intermediate but rather whether the number of ticks in a song is intermediate. The same might be true for the syllable structure of *C. biguttulus* songs in *C. mollis* songs, when an intermediate phenotype is a phenotype with a lower amount of syllable per phrases and not songs containing phrases with a complete syllables structure and phrases without syllables.

In summary, it can be ascertained that male acoustic signals are highly affected by hybridization. The strength of isolation was relatively high, but not complete (0.67 and 0.7, respectively for crossing directions). Playback experiments with original male songs revealed that parental females rarely responded to F1 hybrid songs and Backcross females did this a bit more frequently (Figure 2.4), indicating that F1 males are not behaviorally sterile. It is possible that sexual selection against songs of F1 hybrids males is much weaker for F1 hybrid x F1 hybrid crosses. Nevertheless, natural competitive conditions make it unlikely that F1 hybrid males would find mating partners.

Behavioral isolation of F1 hybrid and Backcross females

Backcross females showed the same preference as *C. biguttulus* females with a reduced response frequency, in contrast to F1 hybrid females which showed no preference at all for any of the artificial male song models. These findings are contrary to previous results on female preference function in F1 hybrids where F1 hybrid females showed preference function similar to those of pure species (von Helversen & von Helversen, 1975b). These authors concluded that the preference functions of both parental species were expressed in hybrids, but that one preference function was dominant over the other, with few exceptions where females showed both parental preference functions (von Helversen & von Helversen, 1975b). There might be multiple reasons for the difference in the observed pattern in my study compared to previous findings. The reduced response frequency relative to other studies also in pure species might be due to two reasons. First, all F1 hybrid females in my preference experiments were tested as soon as they responded to acoustic signals, because after the playback test and after crossing and oviposition they were used for RNA extraction. As I discussed above, the occurrence and motivation of stridulation in females may increase with age and/or with sexual starvation (Butlin & Hewitt 1986). Second, several studies on female preferences excluded females which have not shown a minimum response frequency of 50-60% to at least one test pattern (cf. Einhäupl *et al.*, 2011; Reichert and Ronacher, 2015; Schmidt et al., 2008), which of course increases the mean response level. Unfortunately, detailed information is lacking in the Helversen and Helversen (1975b) paper, but in other papers they mentioned that females were selected by pre–tests with a following sentence comparable to this example *"Approximately 70% of females selected in this manner remained motivated enough to duet through a given testing session"* (cf. Balakrishnan *et al.*, 2001). It remains unclear if females were excluded or not, it was also not possible to extract this information from the presented data, because test trials were often split for visualization.

In addition, asymmetrical integration of the acoustic information in females might cause the loss of the acoustic preference in F1 hybrid females for parental male signals. Clemens et al. (2014) demonstrated that in *C. biguttulus* females the impact of non–attractive units in a male song on response probability is much higher than the impact of attractive units. In F1 hybrid females the preference function of the pure species might be combined (according to von Helversen and von Helversen (1975b) or intermediate. Transferring the

model of Clemens et al. (2014) to hybrid females, then the parental male songs would only match the part of the female preference that comes from the same species. Thus, in this case the other 'heterospecific' part of the female preference would evaluate, with a greater weight, the song units as non–attractive, which would effectively inhibit the female response.

In line with Helversen and Helversen (1975b) the response frequency of F1 hybrid females were slightly different between crossing directions (Figure 2.6 B), indicating that preference is either X linked inherited or that maternal effects are present. Analysis of female response songs revealed similar results in the distribution of phrase/buzz durations like in males with intermediate traits for F1 hybrids and a *C. biguttulus* –like phenotype for Backcross females. The mean response latency of F1 hybrid females was similar to the latency of *C. mollis* females. However peak latency was again intermediate between parental species. *Chorthippus biguttulus* males extract species and sex specific cues from female songs (von Helversen & von Helversen 1983; von Helversen 1997), therefore the latency and the phrase/buzz duration might be important for the male to asses, to localize and to approach the female.

The behavioral isolation barrier seemed to be a strong barrier to reduce gene flow between the species *C. mollis* and *C. biguttulus*, but acts relatively late in the sequence of isolation barriers also indicated by the low absolute contribution of this barrier to the total isolation. RI values did not differ between reciprocal crosses in F1 hybrid, but were substantially different for the direction Backcrosses were compared with. This is not surprising, because F1 hybrids were always backcrossed to *C. biguttulus* and female preference of Backcrosses was similar to the preference of *C. biguttulus* females. Thus, RI values were much higher for Backcross and *C. mollis* comparisons.

The role of reproductive barriers in speciation pathways

The species *C. biguttulus* and *C. mollis* are maintained by multiple pre-and postzygotic (extrinsic and intrinsic) reproductive isolation barriers. The reconstruction of the sequence in which these barriers originated is a major task to understand causes and consequences of the speciation process. In order to understand if reproductive isolation was initiated by extrinsic selection or by intrinsic incompatibility it is important to identify the genetic drivers for reproductive barriers. The prezygotic barriers (based on acoustic and chemical cues) and the extrinsic postzygotic barriers (reduced courtship motivation of hybrid females and behavioral isolation of hybrids) predict that

they evolved as a consequence of divergent sexual selection (Panhuis *et al.* 2001). In addition, experiments on food preference and a genomic divergence analysis with *C. biguttulus* and *C. mollis* found indications for ecological selection (Picaud *et al.* 2003; Berdan *et al.* 2015). Speciation driven by divergent ecological or/and sexual selection can rapidly evolve extrinsic postzygotic and prezygotic barriers and then in later stages intrinsic postzygotic barriers (Coyne & Orr 1989; Orr & Turelli 2001; Seehausen *et al.* 2014). However, I found also very strong intrinsic reproductive isolation between species which theoretically might have evolved earlier than extrinsic post–and prezygotic barriers. Genomic conflict between species is predicted as one driver for Bateson-Dobzhansky-Muller incompatibilities (BDMIs) which lead to intrinsic postzygotic isolation and can cause speciation (Orr & Turelli 2001; Coyne & Orr 2004; Seehausen *et al.* 2014). Transposons are one factor, beside several others, that can cause genomic conflicts. A transcriptomic approach revealed a high transposon activity in *C. biguttulus* and *C. mollis* (Roehr, pers. comm.). In this scenario extrinsic post– and prezygotic barriers may evolve later, which facilitates both ecological coexistence between species and reinforcement of reproductive isolation (Noor 1999; Seehausen *et al.* 2014). Differences in mating signals (Stange 2011, chapter 4) and assortative sperm transfer (Reinhardt 2006) between *C. biguttulus* population might be an indicator for reinforcement, however reinforcement was never explicitly tested. In grasshopper species that form natural hybrid zones the occurrence of reinforcement is predicted as unlikely (Butlin 1998).

Divergent sexual selection, ecological selection and genomic conflict are all potential drivers in the speciation process of *C. biguttulus* and *C. mollis*, but drawing conclusions about the initial force is difficult. However, genes which are involved in prezygotic isolation and are under sexual selection are predicted to have large effects on reproductive isolation because they are directly linked to mating and fertilization patterns and those genes are often highly pleiotropic (Coyne & Orr 2004; Maan & Seehausen 2011; Seehausen *et al.* 2014). The described outlier loci in Berdan et al. (2015) for *C. biguttulus* and *C. mollis* comparisons which could be assigned as candidates in isolation here show pleiotropic patterns in Drosophila and in other animal taxa. This indicates that speciation initiated and driven by divergent sexual selection might be a likely scenario and is a good starting point for further research.

3 Divergence of cuticular hydrocarbon profiles and expression of fatty acid synthases and elongases in *C. biguttulus* and *C. mollis*[2]

3.1 Introduction

Cuticular hydrocarbons (CHCs) are omnipresent on the surface of insects and play a dual role in waterproofing and in chemical communication (Chung *et al.* 2014). In many insect species, CHCs are regarded as a central component of mate recognition systems and thus contribute to behavioral isolation between species (Singer 1998; Ferveur 2005; Howard & Blomquist 2005; Johansson & Jones 2007). Insects have evolved a vast number of CHCs (> 1000) differing in chain lengths, number and position of double bonds and methyl groups, respectively (Martin & Drijfhout 2009; Geiselhardt *et al.* 2011). Comparative studies have demonstrated that CHC profiles tend to be species–specific mixtures ranging in complexity from a couple to more than hundred compounds (Howard 1993; Bagnères & Wicker-Thomas 2010).

The fundamentals of the CHC biosynthesis in insects are well established (Blomquist & Bagnères 2010). The majority of CHCs are synthesized *de novo* in oenocytes by a sophisticated network of fatty acid synthases (FASs), elongases, desaturases, NADPH P450 reductases, and a P450 oxidative decarbonylase (Blomquist & Bagnères 2010; Qiu *et al.* 2012; Chung *et al.* 2014). Methyl-branched CHCs result from the incorporating of methylmalonyl-CoA instead of malonyl-CoA early during chain elongation by a microsomal FAS (Dillwith *et al.* 1982; Chase *et al.* 1990; Juárez *et al.* 1992; Gu *et al.* 1993, 1997; Juárez & Fernández 2007). Despite our basic knowledge about the biosynthesis and composition of many CHC profiles (phenotypes) in a broad range of insect taxa we lack understanding of how new phenotypes may evolve.

[2] Large parts of this chapter will be published in Finck et al. 2016: Divergence of cuticular hydrocarbon profiles in two closely related grasshopper species and the evolution of fatty acid synthases and elongases across insects. *submitted* to Nature Communication

The evolution of novel phenotypes can have different molecular origins (Wagner 2011). Modified gene expression patterns caused by alterations in either *cis*-regulatory sequences or *trans*-acting transcription factors can give rise to novel phenotypes (Gompel *et al.* 2005; Santos *et al.* 2014). In addition, coding sequence changes of preexisting genes and/or gene duplications can also lead to modifications of existing phenotypes. Gene duplications are generally considered as a major source of evolutionary innovations (Lynch & Conery 2000; Zhang 2003; Innan & Kondrashov 2010). Duplication of a gene causes functional redundancy that hampers a stable maintenance of two functional identical genes in the genome(Nowak *et al.* 1997). Consequently, the two paralogs have different evolutionary fates (Lynch & Conery 2000). Most likely, functional redundancy result in pseudogenerization, as one paralog is freed from purifying selection and can accumulate deleterious mutations over time (Lynch & Conery 2000; Zhang 2003). Nevertheless, in some cases, the accumulation of neutral mutations can lead to the origin of novel functions, i.e. neofunctionalization, and the evolution of novel phenotypes. The relative importance of regulatory changes and gene duplications for the origin of species–specific CHC profiles in insects has rarely been investigated.

Here, I use two closely related and morphologically cryptic grasshopper species, *C. biguttulus* and *C. mollis*, to elucidate the molecular mechanisms underlying the divergence of CHC profiles in closely related insect species. These grasshoppers have traditionally been used as model organisms for studying acoustic communication as they produce species–specific calling songs that are reliable signals for species identification (Perdeck 1958; von Helversen & von Helversen 1997; Greenfield 1997; Mayer et al. 2010; Ronacher & Stange 2013). However, several studies suggest that also chemical communication via CHCs can play a crucial role in mate recognition in the genus *Chorthippus* (Ritchie 1990; Butlin 1998, Buckley et al. 2003). Thus, chemical and acoustic communication might be equally important in species and mate recognition in grasshoppers, as already shown for crickets (Orthoptera; Gryllidae) (Simmons 1990; Tregenza & Wedell 1997; Mullen *et al.* 2007; Ryan & Sakaluk 2009; Thomas & Simmons 2010)

In this chapter, I first determined whether CHC profiles (phenotypes) have diverged between sexes and species in *C. biguttulus* and *C. mollis*. Second, I identified candidate genes for FASs and elongases in the *Chorthippus* transcriptomes as these genes are involved in regulation of hydrocarbon

chain length and the position of methyl-branches. Third, I used these candidate genes to examine (i) differential expression patterns between sexes as well as between *C. biguttulus* and *C. mollis* and (ii) estimated coding sequences changes.

3.2 Material and methods

Insects and rearing conditions

For the chemical analyses, *C. biguttulus* was collected at Wendebachstausee near Göttingen, Lower Saxony (N51°28'10.41, E9°56'24.98), and *C. mollis* was collected in Alterlangen, Bavaria (N49°36'35.18, E10°59'3.05) in July and August 2013. For genetic analysis, we used 12 individuals of each species originating from two populations (three males and three females per population), Alterlangen collected in August 2013 and Neuenhagen near Berlin (N52°32'3.33, E13°40'23.01) collected in September 2012 and 2013.

All individuals of *C. biguttulus* and *C. mollis* were caught as late instar nymphs (3rd & 4th) and were subsequently kept in a common room at 25-30°C, 25–30% relative humidity, and a 16:8 h light-dark cycle. Grasshoppers were fed *ad libitum* with a mixture of different grasses (*Festuca rubra rubra, Dactylis glomerata, Poa pratensis*) (seeds from Revierberatung Wolmersdorf Nindorf, Germany). After the final molt, individuals were separated by sex to ensure virginity.

Individuals used for RNA extraction were killed by decapitation within 7 days after their final molt, their gut was removed, and they were stored in liquid nitrogen or in RNAlater (Qiagen, Limburg, Netherlands), due to storage capacity in the liquid nitrogen tank. For RNAlater storage, samples were cut into pieces and incubated in RNAlater at 4°C overnight, the tissue was removed from the RNAlater and stored at -80°C.

Extraction of cuticular hydrocarbons

Grasshoppers were frozen at –20°C four to six days after their final molt. Hydrocarbons were extracted by immersing an individual in 1 ml of *n*-hexane (Rotisolv® HPLC, Carl Roth GmbH+Co.KG, Karlsruhe, Germany) for 5 min. Samples were stored at -20°C until further analysis. Cuticular extracts were concentrated under a gentle stream of nitrogen to a volume of

100 µl. A blank hexane sample was treated the same way to control for potential contamination of samples.

Chemical analysis

In order to examine species or sex specific difference in CHC profile, chemical identification of cuticular extracts was performed on a coupled gas chromatograph-mass spectrometer (GCMS) system (7890A GC – 5975C MSD; Agilent, Waldbronn, Germany). An aliquot of 1 µl of each sample was injected in splitless mode at 300°C. A fused silica column (ZB-5HT Inferno, 30 m × 0.25 mm × 0.25 µm, Phenomenex Inc., Torrance, CA, USA) was used for separation with a constant helium flow of 1 ml per min. The oven temperature program was started at 100°C and then heated to 320°C at a rate of 10°C/min (20 min isotherm). Electron impact ionization was 70 eV.

Hydrocarbons were identified by their mass spectra (Nelson & Sukkestad 1970; Nelson *et al.* 1972; Pomonis *et al.* 1980) and corroborated by their retention indices (Kovats 1965; Carlson *et al.* 1998). Peak areas relative to total peak area were computed for each compound, and peaks that occurred in less than 10 individual CHC profiles were discarded from further analyses. Prior to multivariate statistics, the data were transformed as follows: $z_{ip} = ln[A_{ip}/g(A_p)]$, where A_{ip} is the area of peak i for individual p, $g(A_p)$ is the geometric mean of all peaks for individual p, and z_{ip} is the transformed area of peak i for individual p (Aitchison 1986). As the logarithm is not defined for zero values, a constant of 0.01 was added to each relative peak area (Geiselhardt *et al*. 2012).

Statistical analysis

For quantitative comparisons of the CHC phenotypes, a Principal Component Analysis (PCA) was performed on 34 variables (peaks) and 125 individuals using *FactoMineR* package (Lê *et al.* 2008) in R (R Core Team 2013). By using the PC scores for each individual on PC 1–5 we tested for differences between the two species the sexes and the interaction of species and sex. In total, we ran 5 linear models with the pc scores as dependent variable and species and sex as explanatory variables with the interaction of species x sex in R with the *lm* function. Tukey's HSD post hoc tests were used for pairwise comparisons of males and females within a species and across species (*TukeyHSD*; R). All analyses were performed in R (version 3.2.2).

Identification and ortholog assignment of fatty acid synthases and elongases in Chorthippus

I used a transcriptomic approach to identify candidate genes for CHC synthesis. Based on a literature search, 22 reference protein sequences from *Drosophila melanogaster* related to CHC biosynthesis (i.e. 3 FAS and 19 elongases) were downloaded from FlyBase (http://flybase.org) (Appendix Table B.1). In order to identify homologs in *Chorthippus* grasshoppers, I used tblastn to compare our set of 22 reference proteins to a reference transcriptome of *C. biguttulus* and *C. mollis* respectively (Röhr et al. unpublished). I retained up to 10 hits per protein with a cut-off e–value of 10^{-5}. Best hit transcripts (putative homolog) for each candidate were determined based on highest sequence identity and lowest e–value. Orthologs were then assigned by reciprocal best hits, using the *C. biguttulus* and *C. mollis* candidates.

RNA extraction and sequencing

I wanted to determine if any of the candidate genes were differentially expressed between sexes or species. I collected 12 individuals of each species originating from two populations (three males and three females per population). After approximately 14 days in a standard lab environment the animals were processed. Whole body samples were individually homogenized in TriFast using a MINILYS homogenizer with the Precellys ceramic kit (1.4/2.8 mm) (all from peqlab, VWR International GmbH, Erlangen, Germany). Total RNA was extracted from the samples following the manufacturer's instructions (for peqGOLD TriFast) except that samples that had been stored in RNAlater were precipitated with isopropanol that had been diluted 1:2 with nuclease free water. All total RNA samples were checked for purity and quality using a NanoDrop spectrophotometer (NanoDrop Products, Wilmington, DE, USA) and a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Total RNA samples were determine as pure with a 260/280 value of ~2.0 and a slightly higher 260/230 value associated. If total RNA samples showed strong differences in absorbance, a re-extraction with 1 ml peqGOLD TriFast was performed. All samples showed no visible RNA degradation at Agilent RNA 6000 Pico Assay electropherogram. For mRNA isolation and to decrease ribosomal RNA contamination, mRNA enrichment was performed using the Dynabeads mRNA Purification Kit (Life Technologies, Carlsbad, CA, USA).

For Illumina sequencing, we prepared directional, strand specific RNA libraries using the NEXTflex Directional RNA Seq Kit (dUTP based and NEXTflex RNA-Seq Barcodes, Bioo Scientific, Austin, TX, USA). All libraries showed high quality with a distinct band at approximately 350bp, checked with an Agilent High Sensitive DNA Chip on the 2100 Bioanalyzer and a concentration >10nM. Concentration was measured using a Qubit 2.0 Fluorometer (Life Technologies). Sequencing was performed at the Max-Delbrück-Centrum (Berlin, Germany) on a HiSeq 2000 (Illumina, San Diego, CA, USA) to generate 100-bp paired end reads with a depth of 4–8 libraries per lane. The number of reads per library varied from 5,613,699 - 41,618,214 reads per library (mean 23,361,147). Read numbers were not significantly different between sexes ($F_{1,22} = 1.417$, $p = 0.267$) or species ($F_{1,22} = 0.019$, $p = 0.892$).

Differential expression analysis

After sequencing, I determined if any of the candidate genes were differentially expressed between species or sexes using the trinity differential expression pipeline (Haas *et al.* 2013). Three biological replicates per sex per species (24 total) were used in the Trinity pipeline for differential expression analysis. For abundance estimation, reads from all samples were aligned against the subset of candidate transcripts from the *C. biguttulus* reference using bowtie (Langmead *et al.* 2009). Then, expression values were estimated using RSEM (Li & Dewey 2011). Differentially expressed transcripts were extracted using the *DESeq2* algorithm (Love *et al.* 2014) with a trimmed mean of M-values normalization. Only contigs with a $\log_2$ fold change $>|1|$ and a *P*-value < 0.05 were classified as differentially expressed and *P*-values were corrected for multiple testing (Benjamini & Hochberg 1995). We used counts as dependent variable and species and sex as explanatory variables with the interaction of species x sex. I compared the outcome of the *DESeq2* package with the results of the *egdeR* (Robinson *et al.* 2009) algorithm. Both methods revealed identical differentially expressed contigs, although *P*-values differed. For the sake of clarity, results are shown only for the *DESeq2* algorithm, because this algorithm is more conservative than the edgeR algorithm (Robles *et al.* 2012).

Coding sequence divergence analyses and estimation of substitution rates

In addition, I wanted to test whether our candidate FAS and ELO genes have undergone purifying or positive selection. To do this I estimated rates of

nonsynonymous (dN) and synonymous (dS) substitutions between *C. biguttulus* and *C. mollis*. Based on the tblastn results of *C. biguttulus* and *C. mollis* (see Identification of FAS and ELO orthologs above) I calculated dN and dS substitutions for the FAS and ELO orthologs (Table 3.3) which I had identified before. Reads from all 12 *C. biguttulus* and 12 *C. mollis* (see differential expression analysis above) were pooled by species in silico then aligned to the *C. biguttulus* reference transcriptome (Röhr et al. unpublished). SNPs were called as described in Berdan et al (2015) and used to create two 'species specific' transcriptomes using the *FastaAlternateReferenceMaker* from GATK (McKenna *et al.* 2010). I then used *transdecoder* (part of the *TRINITY* package, Haas *et al.* 2013) to determine Open Reading Frames (ORFs) and estimated dN/dS following the Yang & Nielsen approximate method (Yang & Nielsen 1996) implemented in KaKs Calculator (Zhang *et al.* 2006).

3.3 Results

Composition of cuticular hydrocarbons

The final dataset for the comparison of the cuticular hydrocarbon (CHC) phenotypes of *C. biguttulus* and *C. mollis* comprised 34 different peaks (those that were present in at least 10 individuals; Table 3.1, B.2). The number of peaks per individual was consistent across species and sexes (*C. biguttulus* females: 16.7 ± 1.8 (N = 40); males: 16.9 ± 1.6 (N = 34); *C. mollis* females: 16.1 ± 1.9 (N = 17); males: 16.9 ± 1.1 (N = 34)).

The CHC profiles were mixtures of *n*-alkanes and mono-, di- and trimethyl-branched alkanes (Me-, diMe-, triMeCHCs) with carbon backbones ranging from C_{25} to C_{39}. *N–Alkanes* and methyl–branched alkanes were equally abundant (Table B.2). The *n*–alkane fraction consisted of a homologous series ranging from C_{25} to C_{33}, with *n*–nonacosane (*n*–C29) as dominant compound in both species. In contrast to the uniform composition of *n*–alkanes, both species differed considerably in the composition of their methyl–branched alkanes. In general, the position of the first methyl-branch is shifted by 2 carbon units between the species (i.e., from position 13 in *C. biguttulus* to position 15 in *C. mollis*). Nevertheless, some *C. biguttulus* individuals showed the branching pattern typical for *C. mollis*.

Table 3.1 Factor loadings of each cuticular hydrocarbon peak.
Loading >|0.2| are indicated in bold.

Peak	RT	Compound	PC1	PC2	PC3	PC4	PC5
1	17.60	*n*-C25	0.14	**0.22**	0.19	0.08	0.05
2	19.10	*n*-C27	-0.09	0.14	**0.27**	**0.25**	**0.28**
3	20.51	*n*-C29	0.00	**0.25**	**0.43**	0.07	0.14
4	21.00	3-MeC29	0.10	0.18	0.05	**-0.38**	-0.12
5	21.80	*n*-C31	0.04	**0.21**	**0.46**	0.11	0.02
6	22.00	13-MeC31	0.16	-0.11	-0.01	-0.09	0.11
7	22.45	*n*-C32	-0.04	0.00	0.13	**0.20**	**-0.50**
8	23.14	*n*-C33	-0.06	-0.01	**0.32**	0.11	-0.10
9	23.37	11-/13-/15-MeC33	**0.20**	-0.04	-0.10	**0.22**	0.12
10	23.50	not identified	0.12	**-0.23**	0.09	-0.13	0.10
11	23.54	15,19-/13,x-/11,x-diMeC33	**-0.26**	-0.03	0.01	0.04	0.03
12	23.60	13,19-/11,21-/9,x-diMeC33	**0.23**	0.10	-0.09	0.00	0.03
13	23.82	13,x,x-/11,x,x-/9,x,x-diMeC33	**-0.22**	0.09	-0.07	0.04	-0.04
14	24.19	10-/11-/12-/13-/14-MeC34	0.10	0.03	-0.18	0.01	**0.33**
15	24.42	11,x-/12,x-/13,x-/14,x-diMeC34	**-0.23**	-0.04	-0.06	0.11	-0.06
16	25.09	11-/13-/15-/17-MeC35	0.19	0.00	-0.13	**0.29**	0.14
17	25.34	15,19-/13,17-/11,15-diMeC35	**-0.27**	-0.02	0.01	-0.04	0.11
18	25.40	13,x-/11,23-/9,x-diMeC35	0.19	**-0.27**	0.18	0.00	-0.09
19	25.47	11,x-/9,x-/7,x-diMeC35	0.10	**0.37**	-0.19	0.07	0.02
20	25.58	15,19,x-/13,17,x-triMeC35	**-0.27**	-0.02	0.02	-0.04	0.10
21	25.61	13,17,x-/11,15,x-triMeC35	0.19	**-0.27**	0.19	0.00	-0.09
22	25.64	11,x,x-/9,x,x-diMeC35	0.10	**0.37**	-0.16	0.06	-0.06
23	25.89	3,x-diMeC35	-0.06	0.14	0.04	**-0.44**	**-0.40**
24	26.47	12-/13-/14-/15-/16-MeC36	0.05	**-0.23**	**-0.26**	**0.29**	**-0.22**
25	26.71	not identified	**-0.23**	-0.07	-0.08	0.14	-0.06
26	27.38	11-/13-/15-/17-/19-MeC37	**0.22**	0.10	0.07	0.01	0.05
27	27.71	15,19-/15,21-/13,23-diMeC37	**-0.26**	-0.03	-0.01	-0.01	0.12
28	27.76	13,23-/11,x-/9,x-diMeC37	**0.26**	-0.01	-0.01	0.04	-0.09
29	28.01	15,19,x-/13,17,x-triMeC37	**-0.26**	-0.01	0.03	-0.06	0.10
30	28.05	13,17,x-/11,x,x-/9,x,x-triMeC37	0.19	**-0.26**	0.17	0.01	-0.10
31	28.13	11,15,x-/9,13,x-diMeC37	0.06	**0.27**	-0.10	-0.16	-0.13
32	30.54	i-MeC39	0.08	-0.04	0.00	**-0.28**	**0.23**
33	30.98	15,x-/13,x-diMeC39	0.06	-0.13	-0.04	**-0.34**	**0.29**
34	31.05	11,x-/9,x-diMeC39	0.09	**0.21**	-0.19	0.10	-0.06

Species and sex differences in CHC composition

To assess quantitative differences of the hydrocarbon profiles I performed a principal component analysis (PCA) using the relative composition of the CHC profiles. The first five principal components together explained 71.3% of the total variance in the CHC phenotypes (PC1 = 39.7%, PC2 = 14.5%, PC3 = 8.6%, PC4 = 4.7%, PC5 = 3.9%). PC1 (39.7%) clearly separated the species, while PC2 (14.5%) separated individuals according to sex (Figure 3.1, Table 3.2). This was corroborated by linear models, which showed that PC1, PC3 and PC4 differed significantly between species, while males and females

differed significantly in PC2 and PC3 scores (Table 3.2). We also see significant species x sex interaction in all principal components (Table 3.2).

Table 3.2 Statistics of CHC variation in *C. biguttulus* and *C. mollis* grasshoppers.
Species, sex and the interaction between the two groups were tested by linear models for the principal component (PC) 1-5 with the PC scores as the dependent variable and species and sex as explanatory variables. Shown are the results for PC1-4 (model for PC5 showed no significance). Significant effects are indicated in bold and italics. Total n=125

Effect	PC1		PC2		PC3		PC4	
	$F_{3,121}$	P	$F_{3,121}$	P	$F_{3,121}$	P	$F_{3,121}$	P
Model	247.3	***<0.001***	21.9	***<0.001***	9.8	***<0.001***	5.3	***0.002***
Species	-6.31	***<0.001***	-0.53	0.315	2.00	***<0.001***	-1.01	***0.005***
Sex	0.58	0.078	-3.17	***<0.001***	0.77	***0.035***	-0.34	0.228
Species x Sex	-1.09	***0.042***	1.52	***0.029***	-3.07	***<0.001***	1.70	***<0.001***
Tukey's HSD *post-hoc* test		P_{adj}		P_{adj}		P_{adj}		P_{adj}
C. mollis F x *C. biguttulus* F		***<0.001***		0.745		***<0.001***		***0.024***
C. mollis M x *C. biguttulus* M		***<0.001***		0.118		***0.028***		0.089
C. mollis M x *C. biguttulus* F		***<0.001***		***<0.001***		0.852		0.598
C. mollis F x *C. biguttulus* M		***<0.001***		***<0.001***		***0.044***		0.253
C. mollis F x *C. mollis* M		0.615		***0.014***		***<0.001***		***0.001***
C. biguttulus F x *C. biguttulus* M		0.288		***<0.001***		0.149		0.621

The PC2 interaction is due to a stronger separation between the sexes in *C. biguttulus* and the PC1, PC3, and PC4 interaction is due to that fact that males and females of C. mollis were more strongly separated in comparison to *C. biguttulus* (Figure 3.1, B.1). The compounds that contributed most to PC1 were diMeCHCs (Table 3.1), with negative factor loadings for 15,x-diMeCHCs (indicative for *C. mollis*) and positive factor loadings for 13,x-diMeCHCs (indicative for *C. biguttulus*). The CHC profiles between the sexes differed mainly in the relative amount of triMeCHCs and diMeC35 (peaks 18 and 19). Females exhibited a greater proportion of 11,x-/9,x-/7,x-diMeC35 (peak 19) and 11,x,x-/9,x,x-triMeCHCs (peaks 22 and 31), while males have higher proportions of 13,x-/11,x-/9,x-diMeC35 (peak 18) and 13,x,x-/11,x,x-triMeCHCs (peaks 21 and 30). Similar to the differences between species, the sexes differed mainly in the position of the first methyl-branch of the major CHCs (i.e., shifted by two carbon units between the species).

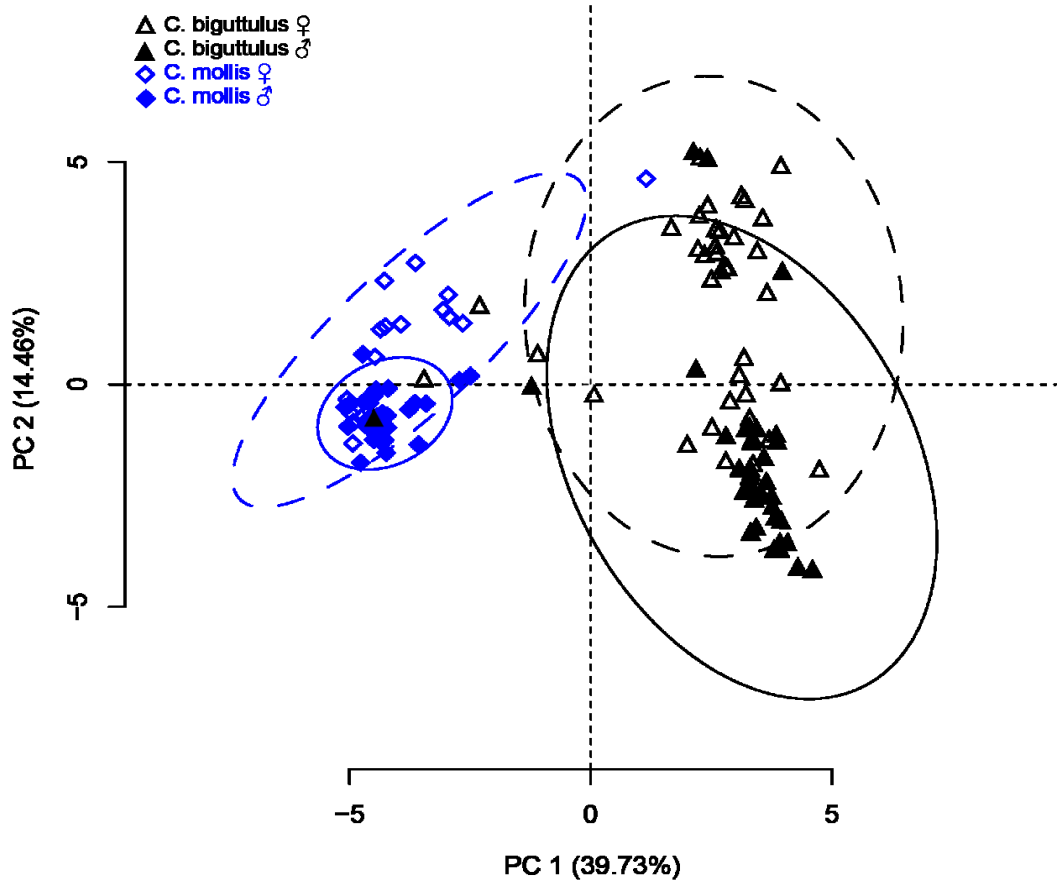

Figure 3.1 Principal component analysis of cuticular hydrocarbon (CHC) phenotypes. Shown are principal component (PC) 1 *versus* 2 with variances explained by each PC given in parentheses. Ellipses indicate 95% confidence intervals. The PCA is based on the relative composition of 34 CHC peaks (see Table 3.1 for loadings)

Ortholog assignment of fatty acid synthases and elongases in Chorthippus

Animal FASs are single multifunctional enzymes consisting of two identical monomers (Chirala & Wakil 2004; Smith & Tsai 2007). The FAS monomer contains seven distinct functional domains in the following order (from the N-terminus): β-ketoacyl synthase (KS), malonyl-/acetyl transferase (MAT), β-hydroxyacyl dehydratase (DH), enoyl reductase (ER), β-ketoacyl reductase (KR), acyl carrier protein (ACP), and thioesterase (TE).

I identified five transcripts coding for putative FASs in both *Chorthippus* reference transcriptomes. The assignment of orthologous genes between both *Chorthippus* species resulted in five ortholog pairs (Table 3.3). The similarities of coding nucleotide and protein sequences, respectively, within ortholog pairs were >98.6% and 99.2%. One ortholog pair (FASG I, Table 3.3) was assigned as ortholog to FASN1 (CG3523) in *D. melanogaster*, while all other FAS

ortholog pairs in *Chorthippus* had no reciprocal best hit with a FAS in *D. melanogaster*.

Table 3.3 Ortholog assignment of FAS and elongase families in Chorthippus

Family	Code[a]	Contig name in reference transcriptome	
		C. biguttulus	*C. mollis*
FAS	FASG I	20030big_male-comp37496_c1_seq1	20003mol_P1-comp71695_c0_seq1
FAS	FASG II-a	20013big_P1_male-comp38343_c0_seq2[a]	20016mol_P1_male-comp81435_c0_seq1
FAS	FASG II-b	20011big_P1-comp52607_c0_seq1[a]	20003mol_P1-comp70825_c0_seq1
FAS	FASG II-c	20011big_P1-comp58522_c0_seq1[a]	20003mol_P1-comp71027_c0_seq1
FAS	FASG III	20030big_male-comp38169_c0_seq1	20164mol-comp17321_c0_seq1
Elo	baldspot	20010big_P1-comp55033_c0_seq1	20016mol_P1_male-comp83867_c0_seq1
Elo	Elo68	20013big_P1_male-comp131546_c0_seq1[b]	20015mol_P1_male_comp119420_c0_seq1
Elo	Elo68	20030big_male-comp106526_c0_seq1[b]	-
Elo	CG33110	20008big_male-comp98995_c0_seq1	20164mol-comp42127_c0_seq1
Elo	CG30008	20013big_P1_male-comp77836_c1_seq1[a]	20007mol_male-comp111352_c0_seq1
Elo	EloF	20030big_male-comp89598_c0_seq1[a]	20015mol_P1_male-comp86102_c0_seq1
Elo	james bond	-	20164mol-comp41288_c0_seq1
Elo	CG5278	20030big_male-comp88504_c2_seq1	20164mol-comp17390_c0_seq1
Elo	CG5326	20030big_male-comp94699_c0_seq1	20164mol-comp45532_c0_seq1
Elo	CG31523	20030big_male-comp91260_c0_seq1	20056mol-comp120587_c6_seq3
Elo	CG31522	20008big_male-comp94799_c0_seq1	20164mol-comp39997_c0_seq1
Elo	CG2781	20030big_male-comp90320_c0_seq1	20007mol_male-comp113584_c0_seq1

[a] No reciprocal best hit to the putative ortholog in *D. melanogaster*.
[b] Identical coding sequences.
[c] No ortholog in other insect orders

The domain structure analysis revealed that only one ortholog pair (FASG I) showed the full open reading frame (ORF) and contained all seven functional domains. The other ortholog pairs lacked certain domains, showed truncated domains or contained incomplete ORFs. Two related ortholog pairs (FASG II -a/c) lacked the MAT domain and another closely related ortholog pair (FASG II -b) has an incomplete ORF that contained only the C-terminal domains. In *C. mollis*, two FAS transcripts with incomplete ORFs (FASG II -b/c) showed short overlapping ends (11 AA) with identical protein sequences, which might be a hint that both transcripts belong to a single gene. The FAS sequences in FASG III lacked the PP domain and showed modification in DH, ER, KR, and TE domains, but not in the KS and MAT domain.

Using the elongase genes from *D. melanogaster*, a tblastn search resulted in 12 transcripts coding for putative elongases in each *Chorthippus* reference

transcriptome, characterized by the ELO domain (PF01151; GNS1/SUR4 family), with a conserved LHXXHH histidine box motif (Hashimoto *et al.* 2008). Both *Chorthippus* species shared 10 ortholog pairs, only two transcripts had no corresponding ortholog in the other species (Table 3.3). In the first case, *C. biguttulus* had two paralogs in the Elo68 cluster while *C. mollis* had only a single copy (Table 3.3). However, the coding sequences of all three transcripts were identical; the 3' non–coding region of the mRNA differed between the two paralogs in *C. biguttulus* and allowed an ortholog assignment of the *C. mollis* transcript. In the second case, *C. biguttulus* lacked the ortholog to CG6921 (james bond).

Signature of selection analysis

I could calculate dN/dS ratios for eight ortholog pairs (Table B.3). Four ortholog pairs showed either no nonsynonymous or no synonymous substitutions, and three sequences had no SNPs. The signature of selection analysis provided no evidence for positive selection acting on FAS and elongases in the two *Chorthippus* species. The dN/dS ratios of ortholog pairs ranged from 0 to 0.129, indicating that purifying selection acts on these genes (Table B.3).

Differential expression of candidate fatty acid synthases and elongase genes

Among the 5 FAS and 10 elongase ortholog pairs of *Chorthippus* species, I found only a single FAS ortholog pair (FASG II-b) that was differentially expressed between both species, with a 2.9-fold higher expression in *C. biguttulus* (Table 3.4). However, the expression levels of this FAS transcript differed not only between species, but also strongly between the sexes (7.6-fold higher in females). In addition, I found two other FASs and three elongases that had significantly higher expression in males than in females (Table 3.4). The two putative FAS transcripts (FASG II-a and FASG III) showed higher expression in males (8.4 fold higher in *C. biguttulus* and 2.4-fold higher in *C. mollis*). The strong differences between the male-biased expression of the FASG II-a transcripts, resulted in a significant species x sex interaction term. Of the three differentially expressed elongases, the CG30008 orthologs showed the strongest male-biased expression (23.2-fold). The other two elongases had 2.3-fold (CG16905) and 3.6-fold (CG5326) higher expression in males.

Table 3.4 Overview of differentially expressed candidate genes[1]

Class	Ortholog cluster	Species[2]		Sex[3]		Species x Sex	
		log2FC±S.E.	P_{adj}	log2FC±S.E.	P_{adj}	log2FC±S.E.	P_{adj}
FAS	FASG II-a			3.08±0.37	<0.001	-1.95±0.46	<0.001
FAS	FASG II-b	-1.52±0.53	0.0347	-2.92±0.53	<0.001		
FAS	FASG III			1.23±0.31	<0.001		
ELO	CG16905 (EloF)			1.20±0.30	<0.001		
ELO	CG30008			4.53±0.49	<0.001		
ELO	CG5326			1.83±0.34	<0.001		

[1] extracted by the DESeq2 algorithm (Love *et al.* 2014)
[2] negative values indicate higher expression in *C. biguttulus*
[3] positive and negative values indicate male- and female-biased expression, respectively.

3.4 Discussion

In addition to their divergent acoustic signals, the sympatric *Chorthippus* grasshopper species, *C. biguttulus* and *C. mollis*, differed significantly in their CHC profiles. The CHC profiles of both species consisted of a series of *n*-alkanes, followed by a series of various methyl-branched alkanes. This study demonstrated that *C. biguttulus* and *C. mollis* as well as males and females of both species show quantitative differences in their CHC phenotypes. Both the general pattern of hydrocarbons with series of *n*-alkanes and methyl-branched alkanes and the interspecific variation based on quantitative rather than qualitative differences seemed to be relatively conserved throughout the family Acrididae (Grunshaw *et al.* 1990; Lockey & Oraha 1990; Chapman *et al.* 1995; Neems & Butlin 1995; Sutton *et al.* 1996). The most striking difference between the two species was the shift of the first methyl-branch position in multimethyl-branched CHCs (i.e., position 13 in *C. biguttulus* and position 15 in *C. mollis*). However, *C. biguttulus* also showed a large variability in the CHC phenotypes, with some individuals exhibiting the methyl-branching pattern typical for *C. mollis*. These individuals clustered together with *C. mollis* in the PCA, illustrating that without this shift, both species are nearly indistinguishable based on their CHC phenotypes. Methyl-branches are incorporated during the fatty acid elongation process by FASs and/or elongases (Blomquist 2010). Thus, I focused on these protein families as candidates for producing the species and sex specific CHC pattern.

Differential expression of fatty acid synthases in Chorthippus *grasshoppers*

Two FAS transcripts (FASG II-a and II-b) in *Chorthippus* showed sex-biased expression but in opposite directions (i.e., male-biased in FASG II-a and female-biased in FASG II-b). In addition the FAS transcript FASG II-b showed indications for differential expression between the species and might be a potential candidate involved in the generation of the divergent CHC profiles of these grasshopper species. The FASG II-a was previously identified in a population genomic scan on *C. biguttulus* and *C. mollis*, indicating that this locus is under selection (Berdan *et al.* 2015). Looking at the coding sequence I found one non-synonymous substitution, but no significant evidence for positive selection (dN/dS = 0.103). The *Chorthippus* sequences of FASG II lack the MAT domain. This domain is responsible for substrate recruitment and loading (Smith & Tsai 2007). Thus, it is unclear whether these transcripts code for functional proteins. However, in *Tribolium castaneum*, an RNAi knockdown of TC15337, that also lacks the MAT domain, leads to a mortality of 60% and 40% after larval and pupal injection, respectively (Dönitz *et al.* 2014). This suggests that TC15337 codes for a functional protein, but it is yet unknown whether it codes for a FAS or another protein.

The FASG III transcript showed female-biased expression. This FAS exhibit modifications in the DH, ER, KR, PP, and TE domains that were either truncated or completely lost. A putative FAS in *T. castaneum* (TC000238) has a very similar domain structure and RNAi knockdown implies that this protein is functional active (100% mortality after larval injection) (Dönitz *et al.* 2014).

Early studies on the fatty acid biosynthesis in insects (De Renobales *et al.* 1986; Blomquist *et al.* 1994; Juárez *et al.* 1996) and vertebrates (Buckner *et al.* 1978; Kolattukudy *et al.* 1987) suggest that a single FAS can synthesize both straight-chain and methyl-branched fatty acids. FASs of the bug *Triatoma infestans* (Hemiptera) (Juárez *et al.* 1996), the housefly *Musca domestica* (Blomquist *et al.* 1994), and the fruit fly *D. melanogaster* (De Renobales *et al.* 1986) can incorporate both malonyl-CoA and methylmalonyl-CoA during chain elongation, resulting in methyl-branched fatty acids. However, a recent study of CHC biosynthesis in *Drosophila* indicates that methyl-branched CHCs are synthesized by a special FAS gene (Chung et al. 2014). The genome of the fruit fly *D. melanogaster* contains three FAS paralogs: FASN1 (CG3523), FASN2 (CG3524), and FASN3 (CG17374). FASN1 is expressed in the fat body, while FASN2 and FASN3 are both expressed in oenocytes of adult flies

(Chung *et al*. 2014). It remains to be tested whether the diversification of methyl-branched CHCs is driven by multiple FAS genes or is a result of the interaction of multiple genes or based on other factors.

Differential expression of elongases genes in Chorthippus *grasshoppers*

The regular FASs release fatty acids with chain length up to 16, with palmitic acid (C16:0) as major product (Blomquist 2010). Thus, the production of long-chained CHCs depends on elongases that elongate the medium-chain fatty acids to very-long chain fatty acids. The elongase family comprises two subfamilies, the S/MUFA and the PUFA subfamily (Hashimoto *et al.* 2008). Members of the S/MUFA subfamily are thought to elongate saturated and monounsaturated fatty acids, while members of the PUFA subfamily elongate polyunsaturated fatty acids. However, this classification is largely based on functional characterization in mammals, whereas the specificity of elongases in insects needs not fit into this classification (Falcón *et al.* 2014).

The expression pattern of elongases was similar in both *Chorthippus* species, but three elongases (EloF, CG30008, and CG5326 orthologs) showed male-biased gene expression. Interestingly, in *D. melanogaster* the EloF (CG16905) gene shows female-biased expression and is involved in the biosynthesis of sexually dimorphic CHC profiles (Chertemps *et al.* 2007). Fruit fly males have CHCs with chain length of C23 and C25 and females with C27 and C29. RNAi knockdown of EloF induced a decrease of C29 dienes and an increase of C25 dienes. The CG18609 gene (EloF) is only expressed in oenocytes and a candidate for the elongation of ω-7 fatty acids from C24 to C26 in male *D. melanogaster* (Wicker-Thomas & Chertemps 2010). In the honeybee, *Apis mellifera*, two elongases, GB54399 and GB40681, are positively correlated with the production of methyl-branched CHCs (Falcón *et al.* 2014). The expression of GB54399 (*james bond* ortholog) is correlated with monomethyl-branched CHCs, while GB40681 (CG30008 ortholog) is highly correlated with dimethyl-branched CHCs (11,15–diMeC27, 9,13–diMeC29, 3,7–diMeC31). Thus, the male-biased expression in the EloF and CG30008 orthologs makes both genes candidates for the biosynthesis of a higher proportion of diMeC35 in males of *C. biguttulus* (3.0-fold) and *C. mollis* (1.7-fold).

Conclusions

I demonstrated that the CHC profiles of the grasshopper species, *C. biguttulus* and *C. mollis,* differ in the first methyl-branch position in multi-methyl-branched CHCs. The high sequence similarity of ortholog pairs and

the absence of positive selection acting on FAS and elongase genes in *Chorthippus* species suggest that the variation in CHC profiles in these closely related species is mainly mediated at the transcriptional level. Similar conclusion can be drawn from the *Drosophila* sister species *D. serrata* and *D. birchii* (Chung et al. 2014). Both species have a functional FASN2 gene, responsible for the biosynthesis of 2-MeCHCs, but *D. birchii* has lost the FASN2 expression in oenocytes, due to cis-regulatory changes. However, the research about the biosynthesis of internally methyl-branched CHCs and its transcriptional regulation is still in its infancy. Although several hundreds of methyl-branched CHCs are known from insects, the enzymatic machinery behind this diversity is largely unknown. In particular, we need a better functional characterization of the FAS and elongase families. Interestingly, insect groups known for a high diversity in methyl-branched CHCs, as ants or beetles, have high numbers of FAS copies. However, further research on the molecular basis of methyl-branched CHCs is necessary to understand the origin of CHC diversity and the role of these genes in speciation.

3.5 The influence of rearing conditions and diets on the cuticular hydrocarbon profiles of *C. biguttulus* individuals

3.5.1 Introduction

Many studies have revealed within species plasticity in CHC profiles by examining condition dependency of CHC biosynthesis. In view of the widespread plasticity that has been found, analysis of CHC profiles should consider that many non-genetic factors may affect the CHC phenotype. Both internal for instance age (Babis *et al.* 2014) or mating status (Thomas 2011), as well as environmental changes, such as climatic changes (Chapman *et al.* 1995) or diet variation (Liang & Silverman 2000). Experimental dietary manipulation in beetles resulted in 80% of the population changing their CHC profile after 14 days on a new host plant (Geiselhardt *et al.* 2012).

The grasshopper species, *C. biguttulus,* is graminivorous with weak food source preferences (Picaud *et al.* 2003; Berdan *et al.* 2015) and is widely dispersed throughout Europe (Heller *et al.* 1998). The entire developmental process in this species, from embryogenesis, nymphal development until sexual maturity depends strongly on climatic factors during rearing, e.g. temperature, humidity or precipitation (Ingrisch & Köhler 1998). Here, I investigate environmental factors (i.e. diet and rearing conditions) which are the most likely factors that differ between habitats and populations. The aim is to examine to what degree those factors also do influence the CHC composition. In order to test this, males and females of *C. biguttulus* were either fed on different diets or were raised under different climatic conditions.

3.5.2 Material and methods

The impact of rearing conditions (lab population versus field population)

I set up a F1 lab population using mated *C. biguttulus* females that were caught in summer 2012 near Göttingen (N51°28'10.41, E9°56'24.98). These insects were kept in mesh polyester cages (47.5 x 47.5 x 93 cm or 47.5 x 47.5 x 47.5 cm, bugdorm Taichung, Taiwan), containing a plastic cup filled with moist granulate (Vermiculite Dämmstoffe, Germany) for oviposition. Eggpods were transferred to petri dishes filled with moist granulate and incu-

bated at 4°C until start of the experiment in August 2013. For the field population *C. biguttulus* males and females were collected, in July 2013, at the same location. Grasshoppers were caught as late instar nymphs (3rd & 4th) and then transferred to the lab and raised under common conditions to exclude all other environmental factors, except the rearing condition in early life stages.

Both lab and field animals were reared for about 14 days until CHC extraction in a single room with a 16:8 h light-dark cycle and maintained at a temperature of 25–30°C with a relative humidity of 25–30%. Field-collected individuals spent only a few days under these standardized lab conditions whereas the F1 lab population experienced standardized conditions during their entire life, from early embryogenesis and diapause until the imaginal stage. After the final molt, individuals were separated by their sex to ensure virginity. Grasshoppers were fed *ad libitum* with only a single grass species (*Festuca rubra rubra)* (seeds from Revierberatung Wolmersdorf Nindorf, Germany) to control for diet as a factor. The biosynthesis of CHCs is continuous so a short time period is sufficient to replace components and change the composition of CHC profiles (Geiselhardt *et al.* 2012). Therefore the lab population and field population differ only in their rearing condition during early embryogenesis, diapause and the first three larval stages.

The impact of different diets on the CHC profile (simple diet vs varied diet)

To examine the impact of different diets on the CHC profile I used individuals collected as late instar nymphs (3rd & 4th) in July 2013 near Göttingen. I divided the animals into two groups; one group (simple diet) was fed *ad libitum* with only a single grass species (*Festuca rubra rubra)* and a second group (varied diet) was fed *ad libitum* with a mixture of different grasses (*Festuca rubra rubra, Dactylis glomerata, Poa pratensis*) (seeds from Revierberatung Wolmersdorf Nindorf, Germany). All grasses were cultured in the same room on Seramis granulate (Seramis GmbH Mogendorf, Germany). As above, grasshoppers were reared for about 14 days until CHC extraction in the same room and under the same conditions as mentioned above.

CHC extraction and chemical analysis (GCMS) was exactly performed as described in Methods 3.2. For quantitative comparisons of the CHC phenotypes, a Principal Component Analysis (PCA) was performed on 40 variables (peaks) with 114 individuals for the diet treatment and 104 individuals for the variation in rearing conditions, using *FactoMineR* package (Lê *et al.*

2008) in R (R Core Team 2013). By using the PC scores for each individual on PC 1-5 we tested for differences between the treatments, sexes and the interaction between treatment and sex. In total, we ran 5 linear models with the PC scores as dependent variable and treatment and sex as explanatory variables. Tukey's HSD post hoc tests were used for pairwise comparisons of males and females within a treatment and across treatments.

3.5.3 Results

To explore the impact of different diets on the CHC phenotype, I tested males and females of *C. biguttulus* which were reared either on a simple diet (single grass type) or on a varied diet (three grass types). Grasshoppers fed with the varied diet showed a higher variability in their CHC composition (Figure B.2, A, B). The diet had a significant impact on PC 1, 3 and 4 (Table B.4). Post-hoc tests showed that females drive this effect while males do not significantly differ (Table B.4). However, the diet treatment did not significantly affect CHC composition in males (Figure B.2 A, B, Table B.4), explaining the treatment x sex interaction found for PC 3.

In line with these results, grasshopper females were more affected by the different rearing condition than grasshopper males (Figure B.3 A, B, Table B.5). The CHC phenotypes of field-collected and lab-reared grasshoppers differed significantly in females on PC 2, 3 and 5. Males differed only weakly on PC 5 (Figure B.3 A, B, Table B.5).

3.5.3 Discussion

These results show that abiotic factors influence the CHC composition in the grasshopper species *C. biguttulus*. This indicates that phenotypic differences in CHC profiles of grasshoppers are not only driven by genetic factors. Diet source and rearing conditions affected the CHC profile in *C. biguttulus*, indicating that environmental conditions during early developmental stages and in later life stages influence the CHC phenotype of the imago. Large geographical and ecological distances between grasshopper populations might also lead to divergence in CHC phenotypes. These results are in line with previous findings that environment interactions can change the composition of CHC profiles (Markow & Toolson 1990; Espelie *et al.* 1994; Etges & Ahrens

2001). Previous studies suggest that plant-derived compounds can serve as precursors for the CHC biosynthesis in phytophagous insects (Pennanec'h *et al.* 1997; Etges *et al.* 2006). For example, the grasshopper *Melanoplus sanguinipes* can incorporate dietary *n*-alkanes into the CHC profile (Pennanec'h *et al.* 1997). Buckley et al. (2003) observed phenotype-environment association on the CHC profile in *C. parallelus* grasshoppers. Further, these authors demonstrated that CHC composition is affected by the vegetation of an environment and by adaptation to variable desiccation stress (Buckley *et al.* 2003). In *C. parallelus* the CHC blend varies between populations and this variation is assumed to be associated with the variation of assortative mating between populations (Tregenza *et al.* 2000a). In some cases, diet-induced changes of the CHC phenotype can lead to behavioral isolation between populations reared on alternative diets (Geiselhardt *et al.* 2012; Najarro *et al.* 2015). However, the different rearing regimes (i.e., diet and rearing conditions) had only a weak effect on the CHC phenotype in *C. biguttulus* and did not eradicate the basic interspecific differences. It remains to be tested whether these changes are behaviorally relevant in *Chorthippus* grasshoppers.

4 Chemical cues from females trigger male courtship behavior grasshoppers[3]

4.1 Introduction

The transmission and recognition of species– and sex– specific cues is crucial for the identification of potential mating partners and the initiation of courtship behavior (Dawkins & Krebs 1978; Andersson 1994). In many birds, frogs and Orthoptera acoustic signals are the most conspicuous courtship signals. Hence, these taxa have been widely used as model systems to study the design and the evolution of acoustic signals and their contribution to reproductive isolation (Kroodsma *et al.* 1982; Greenfield 1997; Gerhardt & Huber 2002). However, courtship sequences often combine multiple signals delivered through multiple sensory channels. Multimodal signals in courtship behavior can reduce the frequency of errors in mating decisions or more effectively indicate the quality of a potential mate than unimodal signals (Moller & Pomiankowski 1993; Johnstone 1996; Candolin 2003; Hebets & Papaj 2005; Simmons *et al.* 2013).

The basic form of mate attraction in grasshoppers, crickets and bush-crickets consists of a phonotactic approach of females towards a singing male (Heller et al. 1998; Gerhardt and Huber 2002). Some species of gomphocerine grasshoppers have evolved a bidirectional acoustic communication system in which both males and females produce and evaluate acoustic signals in order to identify and localize potential mates. In these species, a receptive female responds with a species- and sex-specific song to a male's calling song. The result is an alternating duet during which the male takes a zig-zag approach path to the stationary female (von Helversen 1997). Localizing the female is an important component of mate competition, but close-range courtship is also important because females may still reject males at this stage (Kriegbaum & von Helversen 1992). After encountering the female, the male

[3] This chapter is based on: Finck J, Kuntze J, Ronacher B. 2016. Chemical cues from females trigger male courtship behaviour in grasshoppers. J. Comp. Physiol. A. DOI 10.1007/s00359-016-1081-4

may either attempt to mount her immediately or continue singing, often by producing a softer courtship song. In addition, during courtship a male may touch the female with his antennae. Very excited males sometimes utter a special loud song type, in which the hind legs are raised much higher than during the normal singing movements, immediately before attempting to mount the female (precopulatory movements, see p.64 in Gerhardt and Huber 2002; or 'Anspringlaute' according to Jacobs 1953). These courtship behaviors are important for male mating success. Because of this complex courtship behavior, gomphocerine grasshoppers provide a potentially interesting system to study multimodal signaling. However, in many species, only the acoustic signals produced during long-range mate attraction have been studied.

My focal species are two duetting grasshoppers, *Chorthippus biguttulus* and *C. mollis,* which are morphologically and genetically very similar (Mason et al. 1995; Willemse et al. 2009). Although these two species often occur sympatrically in high densities, hybrids, recognizable by intermediate song patterns, have only rarely been observed in nature (Perdeck 1958; Kriegbaum 1988). Hybridization experiments revealed a strong pre-mating barrier, suggesting a significant role of courtship displays as a prezygotic isolation barrier (von Helversen & von Helversen 1975a; chapter 2).

What are the cues on which mating decisions depend, and which sex decides whether copulations take place? Earlier research on grasshoppers focused mainly on female mate choice and how it contributes to species boundaries (von Helversen & von Helversen 1994; Klappert & Reinhold 2003; Safi *et al*. 2006), but little is known about the selectivity of males. Copulating with an inappropriate partner may incur higher costs for females, due to their high investment in offspring. However, male-female interactions at close range indicate that both males and females are selective in their choice of mates: some males never attempted to mount a conspecific female, even if she had replied to the male's calling song (pers. obs.). Furthermore, in hybridization experiments between *C. biguttulus* and *C. mollis* individuals of both sexes were very reluctant to accept a heterospecific mate (chapter 2).

Several observations indicate that other communication cues in addition to acoustic signals are important for mating success and species discrimination in gomphocerine grasshoppers. In field observations only 50% of *C. biguttulus* pairs that were engaged in acoustic duets actually mated afterwards (Kriegbaum & von Helversen 1992), suggesting that in the remaining

cases the attractiveness of the acoustic signal was either insufficient or other cues determined whether individuals actually mated. In the field, many copulations seem to result from chance contacts between males and females, rather than the directed acoustic orientation described above. In a *C. biguttulus* population with only mute males, 100% of females nonetheless mated, although with a delay compared to females in a population with singing males (Kriegbaum & von Helversen 1992). Similar observations are reported from other species. For example, females of *C. parallelus erythropus* also readily mated with mute males (Ritchie 1990). Nonetheless, acoustic cues are probably important at low population densities, where individuals are less likely to encounter each other by chance. Additional experiments suggest that chemical cues are important for short distance communication of grasshoppers: after removal of the antennae in both sexes, the number of heterospecific matings increased in *C. parallelus erythropus* (Ritchie 1990), and *C. parallelus* males displayed courtship behavior to freshly killed females, but only when the females' cuticular hydrocarbon (CHC) profile was intact (Butlin 1998). In *C. parallelus* chemical analysis revealed that the CHC composition differed between sexes and populations (Tregenza *et al.* 2000a).

Based on these findings I hypothesized that CHCs are involved in close range chemical communication and may affect courtship behavior. More specifically, I can ask whether the acoustic (long distance) signals are necessary and perhaps sufficient to guarantee species separation and sex recognition, or whether additional close-range CHC cues are also relevant. Cuticular hydrocarbons evolved in insects primarily as a physical protection barrier against desiccation, but in many insects they also have important signaling functions in inter- and intraspecific communication (Hadley 1989; Singer 1998; Howard and Blomquist 2005). If multiple signaling modalities are involved in the courtship sequence of these animals, then we predict that chemical cues, in addition to acoustic cues, should allow males to detect the species identity and sex of potential partners. In chapter 3, I showed that the CHC profiles in *C. biguttulus* and *C. mollis* exhibit species- and sex-specific differences, which is a prerequisite if CHC cues are to be used as conspecific mating signals.

In this chapter I addressed two questions. First, I asked whether males of *C. biguttulus* and *C. mollis* use an additional close range communication channel to discriminate between conspecific vs. heterospecific mating partners. Second, I asked how chemical cues of conspecific and heterospecific

females affect male courtship behavior. Reinhardt (2006) observed that *C. parallelus* males transfer less sperm to females from an allopatric population than to sympatric females, and thus are able to discriminate between the two. Thus, I also tested whether males discriminate against conspecific females from a distant population exclusively on the basis of CHC cues. We developed a bioassay, with some similarities to the method of Gray et al. (2014), which allows for a rigorous test of the effects of chemical (olfactory) cues, excluding visual, acoustic, tactile and vibratory cues. By presenting a piece of filter paper soaked with various CHC extracts we show that chemical signals suffice to allow grasshopper males to identify species and sex of potential mates.

4.2 Material and methods

Grasshopper identification and collection sites

We collected males and females of *C. biguttulus* and *C. mollis* from a population near Berlin, Germany (N52°32'3.33; E13°40'23.01) and collected additional *C. biguttulus* individuals from a population near Göttingen, Germany (N51°28'10.41, E9°56'24.98). All grasshoppers were caught between July and September 2014. Males were identified in the field based on species-specific songs and were kept separately in the lab by species and population in mesh polyester cages (47.5 x 47.5 x 93 cm or 47.5 x 47.5 x 47.5 cm, bug dorm Taichung, Taiwan) at room temperature. They were fed *ad libitum* with fresh grass and fish food (TetraMin *Hauptfutter für alle Zierfische*; Melle, Germany).

Cuticular hydrocarbon samples

I prepared three CHC samples: one sample from *C. biguttulus* females (from Göttingen) one sample from *C. mollis* females (from Berlin) and one sample from *C. biguttulus* males (Berlin). In order to test for species recognition we presented the CHC samples from *C. biguttulus* and *C. mollis* females to conspecific and heterospecific males. In addition, the CHC sample from *C. biguttulus* females (Göttingen) was also presented to conspecific males from Berlin, to test for population differences (see Table 4.1). The third CHC sample, obtained from *C. biguttulus* males from Berlin, was used to test for sex discrimination with *C. biguttulus* males (from Berlin).

Extraction of cuticular hydrocarbons

For each CHC sample ten grasshoppers were caught in the field using glass vials and were immediately flash frozen on dry ice and then transferred to the lab and stored at -20°C until further processing. Identification of female *C. biguttulus* in Göttingen was possible using visual characteristics, as no morphologically similar grasshopper species occur at this location. Because both *C. biguttulus* and *C. mollis* occur sympatrically in the area surrounding Berlin, we identified males by their species-specific song characteristics and females using species-specific wing characteristics (Bellmann 1993). For documentation, we cut one fore wing from each female before storage.

The three CHC samples were each made by immersing ten individuals in 10 ml of n-hexane (Rotisolv® HPLC, Carl Roth GmbH+Co.KG, Karlsruhe, Germany) for 5 min. The samples were concentrated to 4 ml by evaporation at room temperature and then stored in glass vials (Rotilabo 4 ml; Carl Roth GmbH + Co.KG, Karlsruhe, Germany). Samples were stored in a refrigerator at 4 °C.

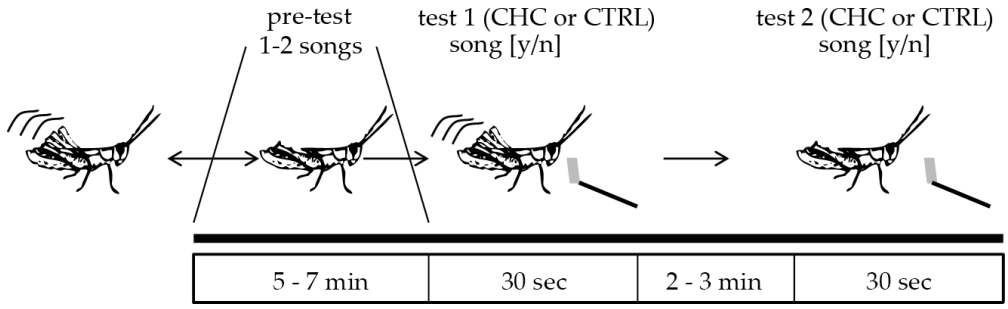

Figure 4.1 Schematic illustration of the bioassay (Test paradigm).

Bioassay

For the behavioral tests we used *C. biguttulus* males from Berlin and Göttingen and *C. mollis* males from Berlin. Sexually motivated males can be recognized by the production of calling songs. As mentioned in the introduction, males sometimes produce a special type of song immediately before attempting to mount a female, indicating their highest motivation to mate ('Anspringlaute' Jacobs 1953, precopulatory movements). This type of song was also observed in some of our test males in response to the presentation of female CHCs. This behavior provides additional support that the bioassay

test is well suited to investigate the role of CHCs in close range courtship behavior.

Spontaneously singing males were transferred to a glass plate (38 x 34 cm) framed on three sides with mesh. Males were allowed to adapt to the setup for 5–7 min before the test trial started. In order to be tested, males had to produce at least one song during the adaptation period but not more than one song within 2.5 min. This was important to assess the motivation level of the test male and the rate of spontaneous singing activity. We excluded stressed males that showed avoidance reactions, such as escape behavior, and males with a high spontaneous calling song rate, because this would have prevented a reliable distinction between spontaneous activity and a response to the CHC stimulus. Every male who conformed to these stipulations was subject to two test trials, one test with a CHC stimulus and one with a control stimulus. The order of stimulus presentations was chosen randomly and balanced between individuals. For each test trial 10 µl of CHC sample solution or of the solvent n-hexane (as the control) was pipetted onto a 1 cm^2 piece of filter paper (MN 615, thickness 0.16 mm, Carl Roth GmbH+Co.KG, Karlsruhe, Germany) and allowed to dry for 1.5 min at room temperature followed by 30 seconds under a heat lamp (Phillips IR 150, Korea). The filter paper was placed close to, but not in physical contact with, the male (0.5-1 cm range). However, most males soon touched the filter paper with the antennae or even walked over it. If a male sang at least once within 30 seconds after stimulus presentation it was considered as a positive response; if no song occurred within this time this was considered as a negative response. After 30 seconds the filter paper was removed and we waited 2–3 min before presenting the second stimulus. Each male was tested only once and the order of stimuli was randomized between individuals (see Results). For a schematic illustration of the bioassay see Figure 4.1. The temperature for the tests was maintained at 30 ± 2°C using two lamps (Sun GLO, 100w Exo Terra, Holm, Germany and Phillips IR 150, 150 w, Korea) to simulate natural conditions during the mating season.

We classified an individual's behavior into four different categories (Table 4.1): **A**: no response to either of the stimuli, **B**: a response to the CHC stimulus (CHC +) but not to the solvent control (CTRL -), **C**: the opposite behavior, a response to the control (CTRL +) but not to the CHC stimulus (CHC -), and **D**: a response to both stimuli. In order to test whether the species differ in response latency to a stimulus, the duration between the start of

filter paper presentation and the beginning of the response song of *C. biguttulus* and *C. mollis* males was measured for response pattern B.

Statistical analysis

In order to test whether males perceive and use chemical signals as species and sex recognition cues, the statistical analysis was focused on the capability of a male to detect and distinguish between a chemical signal and a control stimulus. Thus, I use McNemar's mid-p test (Fagerland et al. 2013) to test for the difference in the occurrence of calling responses to CHC stimulation and the control. Population differences in response pattern between males from Göttingen and males from Berlin were tested using a two-sided Fisher Exact test. Differences in response latencies between species for response pattern B were tested with a two tailed Wilcoxon-Mann-Whitney-Test. The effect of presentation order on response pattern was tested with a two-sided Fisher Exact test. All tests were calculated in R (R Core Team 2013).

4.3 Results

The presentation order of the stimulus (CHC first vs control first) was balanced between individuals and had no effect on the response pattern (Fisher's exact test for *C. biguttulus* males to conspecific female CHC: Göttingen $p = 1$; Berlin $p = 0.76$, and for *C. mollis* males to conspecific female CHC: $p = 0.35$). Among the four possible response patterns (see Table 4.1), the categories B) – calling song response to the CHC stimulus but no response to the solvent control (CHC+/CRTL-) – and C) – no response to the CHC but response to the control (CHC-/CTRL+) – are most meaningful. The most common response (61.1% of all tested males) was for *C. biguttulus* males to respond to the conspecific female-CHC sample (obtained from females of their own population), but not to the solvent control stimulus (Figure 4.2 I Species recognition *C. biguttulus*; category B in Table 4.1). A McNemar mid-p test showed that this effect was statistically significant (χ^2 1 = 9.48, $N = 36$, $p < 0.001$). In contrast, the other response patterns occurred much less frequently: A: 13.9%, C: 13.9%, and D: 11.1% (Table 4.1). The population of origin also affected the response likelihood towards the conspecific female CHC sample: males from Göttingen tested with the female CHC sample from the same population responded at a higher rate than did the males from a distant population (Berlin) to the same stimulus (Figure 4.2 I, comparison of the two populations: Fisher's exact test, $p = 0.015$). Nevertheless, the female CHC sample from Göttingen clearly excited the males from Berlin (χ^2 1 = 8.1, $N = 25$, $p < 0.001$, Table 4.1). To test for species recognition we examined responses to a heterospecific female CHC sample: *C. biguttulus* males (from Göttingen) responded equally rarely to a heterospecific female CHC stimulus as to the control stimulus (Figure 4.2 II; χ^2 1 = 0, $N = 20$, $p = 0.625$).

Table 4.1 Response pattern of test males to conspecific & heterospecific female CHC samples.
To test for sex recognition, the responses of males to a CHC sample from conspecific males are also shown. To test for population discrimination, *C. biguttulus* males from Berlin were tested with the female CHC sample from Göttingen. The columns denoted (A) through (D) denote the different categories of male response based on whether they gave a response song (+) or did not respond (-) to the respective stimulus (CHC or control CTRL). The origins of grasshoppers are indicated by superscript letters g and b for Göttingen and Berlin, respectively.

Stimulus	Test males	(A) CHC-/CTRL-	(B) CHC+/CTRL-	(C) CHC-/CTRL+	(D) CHC+/CTRL+	$\sum$	McNemar mid-p test
C. biguttulus ♀^{g}	*C. biguttulus*^{g}	5	22	5	4	36	< 0.001
	C. biguttulus^{b}	11	10	0	4	25	< 0.001
	C. mollis	8	2	2	8	20	1
C. mollis ♀^{b}	*C. mollis*^{b}	4	17	2	7	30	< 0.001
	C. biguttulus^{b}	14	2	1	3	20	0.625
C. biguttulus ♂^{b}	*C. biguttulus*^{b}	14	1	2	3	20	0.625

g Göttingen
b Berlin

Chorthippus mollis males exhibited a similar response profile as *C. biguttulus* males: 56.7% B-responses and much lower occurrence rates for the other response patterns (13.3%, 6.7%, and 23.3% for behavior patterns A, C and D, respectively; Figure 4.2 II Species recognition *C. mollis* and Table 4.1). A McNemar test validated this effect (χ^2 1 = 10.32, N = 30, p < 0.001). The latency of responses to conspecific female odor was not significantly different between the species (Wilcoxon-Mann-Whitney-Test U = 244, N1 = 17 N2 = 22, p = 0.11). I again examined responses to heterospecific CHC samples in order to test for species recognition. *C. mollis* males responded equally rarely to the female *C. biguttulus* CHC stimulus and the control (Figure 4.2 II; χ^2 1 = 0, N = 20, p = 1).

As an additional test for sex recognition *C. biguttulus* males were exposed to conspecific male CHCs. The response frequency to the conspecific male CHC sample was very low, and similar to the response frequency to the control stimulus (Figure 4.2 III Sex recognition, (χ^2 1 = 0, N = 20, p = 0.625), indicating that males discriminated between sexes based on CHC cues).

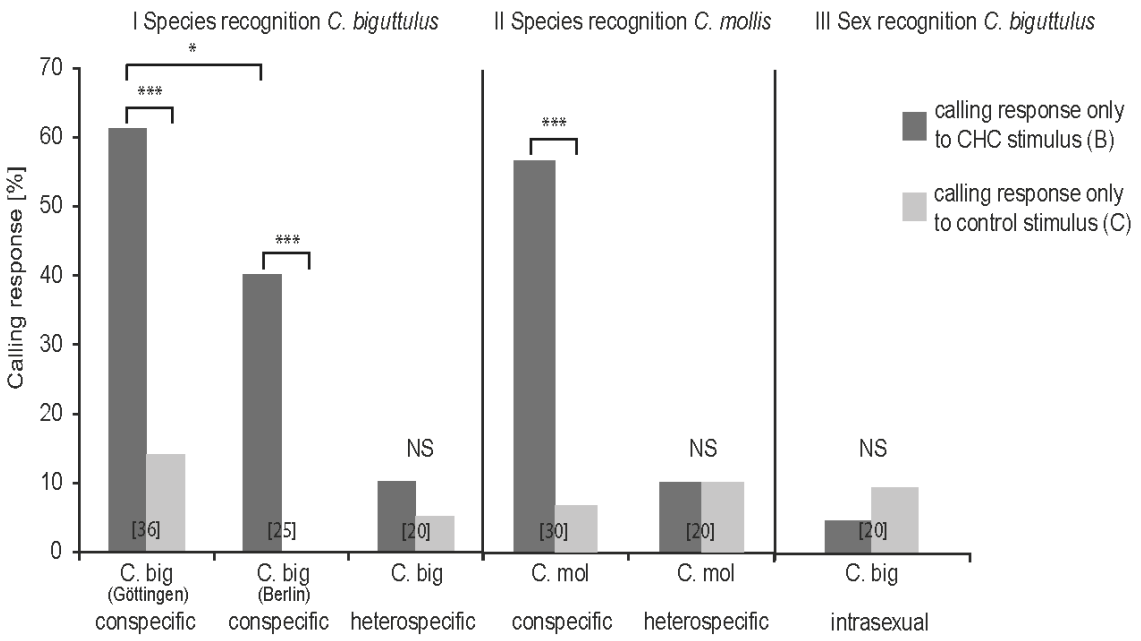

Figure 4.2 Response frequencies of test males [%] to the three CHC samples.
Positive responses to the CHC stimulus in combination with negative responses to the control stimulus (CTRL) (pattern B) are shown as dark grey bars (see Table 4.1). The opposite pattern C (negative to CHC together with positive to CTRL) is shown as light grey bars. **I)** Response frequencies of *C. biguttulus* males (C. big) from Göttingen and Berlin to the *C. biguttulus* female CHC sample from Göttingen (conspecific), and heterospecific response frequencies of *C. biguttulus* males from Berlin to the *C. mollis* female CHC sample (from Berlin). The origin of test males is given in round brackets. **II)**. Response frequencies of *C. mollis* males to the *C. mollis* female CHC sample (conspecific) and to *C. biguttulus* female CHC sample (heterospecific). **III)** Response frequencies of *C. biguttulus* males to the conspecific male CHC sample. The sample size for each test group is given in square brackets. Significance levels: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

4.4 Discussion

The results presented above provide strong evidence that male *C. biguttulus* and *C. mollis* use chemical cues for species recognition at close range. Furthermore, tests with *C. biguttulus* males showed that chemical cues can also serve as a sex recognition cue. The cues derived from the CHC profile of females were sufficient to induce courtship behavior in conspecific males with a general readiness for mating. Therefore, acoustic, vibrational or visual stimuli were not essential for males to obtain species- and sex- specific information about potential mates. Thus, chemical cues complement acoustic long distance signals and contribute substantially to courtship behavior in grasshoppers.

High selectivity of males

The results also bear on the question of whether the selection of a mating partner of the correct species is performed mainly by the females, or whether there is also male mate choice. It has been assumed that copulating with a heterospecific mate would incur only small costs for males, whereas it may waste a large portion of a female's lifetime reproductive effort. Thus, mate choice is assumed to be performed largely by females (Darwin 1871; Kriegbaum 1988; Andersson 1994). However, increasing evidence suggests that male mate choice is also common, even when the males provide no parental care (Bonduriansky 2001; Edward and Chapman 2011).

Which factors contribute to male choosiness? According to Bonduriansky (2001) the major factors that may promote the evolution of male mate choice are (i) high male mating investments, and (ii) large variance in female quality (e.g. fecundity, reproductive condition). There is no evidence for a substantial mating investment of male grasshoppers in the form of direct benefits like nuptial gifts transferred to females (Reinhardt and Köhler 1999; Klappert and Reinhold 2007). However, spermatogenesis in grasshoppers takes some time and males need 2 to 3 days to fully recover their mating capabilities (Reinhardt 2007; Wirmer et al. 2010). In addition, intrasexual competition between males may increase their courtship activity, resulting in a higher singing activity or increased searching behavior. Edward and Chapman (2011) argued that a higher investment into mating effort (e.g. higher courtship activity) will increase the ability to meet receptive females, but will also reduce the capacity of males to mate with a high number of females.

Hence, similarly to the effects of higher investment in parental care, this increase in mating investment could drive the evolution of male mate choice (Edward and Chapman 2011). Grasshopper females tend to mate several times (Kriegbaum 1988; Reinhardt and Köhler 1999; Wirmer et. al 2010). A male can therefore expect to meet a number of receptive females during its lifetime, and the acoustic long-distance communication may serve to reduce search costs. In *C. biguttulus* the predation risk during the phonotactic search for females appears to be a major cause of the lower survival rates for males than females in the field (Kriegbaum 1988).

Male choosiness would also be beneficial if females show quality differences in fecundity or reproductive condition. Indeed, the quality of *C. biguttulus* and *C. mollis* females appears highly variable, due to individual differences in ovariole numbers and body weight (unpublished data), as well as in the amount of eggs per egg-pod, egg weight and egg size (Kriegbaum 1988, 1997; Thorens 1989). Hence, males may increase their reproductive success by choosing larger females that indicate higher fecundity (for other species see Bonduriansky 2001; Servedio 2007; Edward and Chapman 2011). *Chorthippus parallelus* males transfer more sperm to sympatric females than to females of an allopatric population belonging to a different subspecies (Reinhardt 2006). This result demonstrates male choosiness and indicates that the costs of sperm production are not negligible. We observed a reduced calling response of *C. biguttulus* males to CHCs of conspecific females from a distant population (Berlin in Figure 4.2 I). This also indicates a preference for females from the same population, although, unlike *C. parallelus* (Reinhardt 2006), in *C. biguttulus* there is no evidence for incompatibilities between the two populations. Nevertheless, it is conceivable that mating with a partner adapted to the local habitat may be advantageous. Interestingly, in a transfer experiment between two populations, *C. biguttulus* females showed no preference for the acoustic signals of males from the same population - unfortunately only the response to acoustic signals has been tested (Klappert and Reinhold 2005).

Rejection of heterospecifics probably represents avoiding the 'bad end' in a continuum of quality cues (Safi et al. 2006). A heterospecific partner belongs to the cohort of extremely low-quality mates, but strong quality differences may also exist among conspecifics. These quality differences have been investigated mostly from the viewpoint of females, but Reinhardt's (2006) and the results indicate that these differences may be relevant also for males.

Cuticular hydrocarbons as signals

Cuticular hydrocarbons of many insects have important signaling functions in inter- and intraspecific communication (Hadley 1989; Singer 1998; Howard & Blomquist 2005). The majority of CHC components found in grasshoppers were characterized by relatively long carbon chain lengths, consisting of mixtures of *n*-alkanes and mono-, di- and trimethyl-branched alkanes with carbon backbones ranging from C_{25}–C_{39}. These chain lengths suggest that most components of the CHC profile are non-volatile (Neems & Butlin 1994; chapter 3), although at very close range some components may also be volatile (Saïd *et al.* 2005; Farine *et al.* 2012). Cuticular hydrocarbons are often effective as contact pheromones, due to their non-volatility (Tregenza & Wedell 1997; Ginzel *et al.* 2003). However, some males (< 10%) showed correct signal detection without direct contact with the paper. This suggests that these males either happened to sing spontaneously, or that volatile components effective at close range were responsible for the positive response (see Saïd et al. 2005; Farine et al. 2012).

The mating status may influence the CHC profiles as has been shown in ants, bees and fruit flies (Blomquist *et al.* 1998; Howard & Blomquist 2005; Thomas 2011). Since the females for the CHC samples were taken from the field, we do not know their actual mating status; likely most females were not virgin (cf. Kriegbaum & von Helversen 1992). However, we used only a single sample (mixed from 10 *C. biguttulus* females), and similarly a single sample from 10 *C. mollis* females, for all tests. Thus, a conceivable difference in the mating status composition between the two species samples should have been revealed in the results, where I did not find any indications for such an effect.

Advantages of multimodal signaling

The basic form of acoustic communication in grasshoppers involves singing males and females that perform a phonotactic approach towards the signaler. In this scenario olfactory cues provided by the female are suitable signals for the male to evaluate an approaching mating partner. Chemical communication may also help to distinguish between mated and unmated females by allowing for the detection of male CHCs from previous matings (see e.g. Bonduriansky 2001, p 323). In addition, multiple cues are thought to reduce the costs of mate choice (Backwell & Passmore 1996; Candolin & Reynolds 2001). These authors argue that the first cue indicates a potentially suitable mating partner (i.e., of the correct species and sex), while the second cue is a supplemental indicator for mate quality. Multiple messages may provide more information about the quality of mating partners and also reduce the time costs for mate inspection (Candolin 2003; Hebets & Papaj 2005). For instance, in the cricket *Teleogryllus oceanicus,* females use both acoustic signals and male CHCs for mate choice. Acoustic signals convey information about the genetic quality and species identity of the male, whereas the CHC profile provides information on genetic similarity and thus compatibility with an individual's own genotype (Tregenza *et al.* 2006a; Thomas & Simmons 2011; Simmons *et al.* 2013). Remarkably, the long-distance calling songs of *C. biguttulus* males may already convey information about the quality and health of the signaler (Stange & Ronacher 2012). It would therefore be particularly interesting to investigate what additional information may be transferred via chemical cues in grasshoppers. The fact that CHC signals were sufficient to elicit precopulatory movements ('Anspringlaute' Jacobs 1953, see methods) in some males further underlines the relevance of CHC signals for mating decisions.

Conclusions

The results show that CHC components of conspecific females provide sufficient cues to induce specific courtship behavior in grasshopper males. They further demonstrate male choosiness and indicate that male *C. biguttulus* and *C. mollis* use a previously neglected communication channel to assess potential mates. More research is now needed to estimate the relative impact of acoustic signals and chemical signals on mate choice, and the role of male mate choice in response to conspecific females of different quality. The bioassay is very suitable to test whether male mate choice is present in this system,

and which factors might have favored the evolution of male mate choice. The principle of this bioassay might be extended to other species to investigate olfactory behavior in multimodal communication systems.

5 Discussion and conclusion

In this thesis, I identified multiple reproductive isolation barriers between two closely related species, *C. biguttulus* and *C. mollis,* and quantified their respective contributions to isolation. In addition, I used an integrative approach, involving behavioral chemical and genetic analyses, to explore a previously neglected communication channel in these species. I conclude that, chemical signals are crucial in the maintenance of species isolation. Genes that are associated with chemical signaling are also good candidates to be involved in the initial speciation process. I will now discuss several speciation scenarios and the implication of my results in a broader context.

The prezygotic barriers in *C. biguttulus* and *C. mollis* are currently the strongest impediments to gene flow. However, I found that there are also strong extrinsic and intrinsic postzygotic isolation barriers and it should be emphasized that the fact that postzygotic barriers act later in the life cycle than prezygotic barriers does not necessarily mean that they were insignificant during the initial process of speciation (Coyne & Orr 2004). The drivers for the evolution of prezygotic and extrinsic postzygotic isolation differ from those for the evolution of intrinsic postzygotic isolation. Prezygotic and extrinsic postzygotic isolation can evolve by ecological or sexual selection, while intrinsic isolation may evolve by genetic drift or through genomic conflict (Coyne & Orr 2004; Seehausen *et al.* 2014). However, the population/species signatures can look very similar, especially when additional reproductive barriers evolve after species formation is completed (Table 5.1).

Table 5.1 Prediction of population/species signatures in different speciation scenarios modified after (Safran *et al.* 2013; Seehausen *et al.* 2014)

Speciation driven by	Population/Species signature	Reproductive isolation based on
Sexual selection	Ecology similar, sexual signals are different Mate preference are based on divergent sexual signals	Behavioral reproductive isolation; prezygotic & extrinsic postzygotic barriers, intrinsic postzygotic barriers may evolve later
Sexual selection and ecological selection	Ecology different & sexual signals are different, Divergent sexual signals co-vary with ecological context	Behavioral reproductive isolation & ecological isolation is possible; prezygotic & extrinsic postzygotic barriers, intrinsic postzygotic barriers may evolve later
Negative epistatic interactions (BDMIs) unlikely for scenarios of sympatric speciation	Various signatures are possible	Fitness reduction in hybrids, Intrinsic postzygotic barriers evolve first, prezygotic & extrinsic postzygotic may evolve later

Species divergence initiated by selection can accumulate in the presence or absence of gene flow, whereas speciation driven by intrinsic barriers is thought to be unlikely in the presence of gene flow (Gavrilets 2004; Seehausen *et al.* 2014). Intrinsic isolation arises most frequently from negative epistatic interactions (i.e., Bateson-Dobzhansky-Muller-incompatibilities (BDMIs)) and can be driven by various factors. Intrinsic postzygotic isolation was strong between *C. biguttulus* and *C. mollis*, which indicate either that speciation was driven by negative epistatic interactions or that these species are relatively advanced on the speciation continuum (chapter 2). If speciation is driven by intrinsic isolation, prezygotic and extrinsic postzygotic barriers can evolve later (Seehausen *et al.* 2014). As a consequence the prezygotic and extrinsic postzygotic barriers then would allow ecological coexistence of species and reinforcement of reproductive isolation. In this scenario the population/species signature would look similar to a speciation scenario, which was driven by sexual selection and where intrinsic postzygotic isolation barriers have evolved later (Seehausen *et al.* 2014, Table 5.1). In general, prezygotic and extrinsic postzygotic isolation are predicted to evolve faster than intrinsic postzygotic isolation (Coyne & Orr 1997; Orr & Turelli 2001). Nevertheless, the possibility that isolation between *C. mollis* and *C. biguttulus* arose by genetic incompatibilities provides an alternative to speciation scenarios that

are driven by prezygotic and extrinsic postzygotic isolation, if it is assumed that speciation was allopatric.

Earlier work on speciation in gomphocerine grasshoppers assumed ecological selection (Butlin 1998) or sexual selection on acoustic mating traits as the main driving force for species divergence (Mayer *et al.* 2010; Vedenina & Mugue 2011). Vedenina and Mugue (2011) hypothesize that the increase in complexity of acoustic courtship signals between related species was driven by sexual selection and led to speciation. However, these authors further assumed that the recent radiation in the genus *Chorthippus* was driven by other forces than sexual selection on acoustic mating traits, because of the ancestral mating signal (i.e., lack complex courtship songs) in this genus (Vedenina & Mugue 2011). The experiments in the second chapter demonstrated that, in addition to acoustic signals, several other barriers contribute to reproductive isolation between the closely related species *C. biguttulus* and *C. mollis*. In addition, my results suggest that chemical cues are involved in mating behavior in these species and that the mating traits are multimodal (i.e., involving acoustic and chemical cues). Mating traits are often driven by sexual selection and there is much controversy over the conditions in which sexual selection can act as driving force in speciation (Andersson 1994; Ritchie 2007; Smadja & Butlin 2011; Safran *et al.* 2013; Servedio 2015). One factor that affects the probability that sexual selection leads to speciation is the genetic architecture of mating traits. Theoretical models on sexual selection in speciation events assume that sexual traits are controlled by a few loci (Servedio *et al.* 2011), i.e. simple genetic architecture (Gourbiere 2004). A polygenic genetic architecture (i.e., complex genetic architecture) is assumed to even act against speciation in sympatry (reviewed in Ritchie, 2007). The genetic architecture of acoustic mating traits is predicted to be complex, including multiple genes on multiple loci (Ritchie & Phillips 1998). In contrast to acoustic mating traits, chemical mating traits are typically characterized by simple genetic architecture which facilitates rapid evolution (reviewed in Smadja and Butlin, 2009). My results indicate that chemical cues are involved in reproductive isolation, which increases the likelihood that sexual selection was important for speciation in these two species.

Interestingly, the differences in CHC composition between *C. biguttulus* and *C. mollis* were basically mediated by a shift of the first methyl-branch position in multimethyl-branched CHCs (chapter 3). Although this difference is small, behavioral tests clearly showed strong preference for conspecific

CHC blends (chapter 2, 4). My results are in line with many studies in various insect taxa, such as flies, bees, beetles and walking sticks (reviewed in Smadja and Butlin 2009). These studies demonstrated that CHC phenotypes in closely related species often differ only by minor changes of component structure or by changes in the ratio of component production. These changes are often based on only a few or even single genes (Coyne & Orr 2004; Smadja & Butlin 2009). For my focal species the divergence in CHC profiles between the two species might have been driven by the differential expression of a single fatty acid gene (chapter 3). Al-Wathiqui et al. (2014) found evidence that reproductive isolation between two recently diverged Lepidopteran strains is based on differential expression. This indicates that expression differences in genes can contribute to reproductive isolation and species divergence. An additional FAS ortholog was identified as an F_{st}-outlier in a population genomic scan (Berdan *et al.* 2015), indicating that this gene is under selection. The coding sequence in this outlier had one non-synonymous substitution between *C. biguttulus* and *C. mollis* individuals (Berdan et al., 2015, chapter 3). Although I found no evidence for positive selection for this locus, a single non-synonymous substitution can result in new phenotypes. Therefore, this FAS ortholog is another candidate which may also contribute to CHC diversity between species.

To summarize, chemical traits often play significant roles in speciation, and the evolution of new phenotypes is facilitated by the genetic architecture. However, the role of sexual selection as the initial selective force leading to speciation is controversial (Panhuis *et al.* 2001; Ritchie 2007; Nosil 2008; Smadja & Butlin 2011; Safran *et al.* 2013; Servedio 2015). Ritchie (2007) proposed that sexual selection as the only evolutionary force for species divergence in sympatry is rather unlikely and that sexual selection presumably often acts alongside ecological selection or selection for species recognition.
In many insect species the CHC profile is affected by the environment (e.g. climate conditions or diet) and thus can act as a signal of ecological performance (for *Chorthippus*: Buckley *et al.*, 2003; Neems & Butlin, 1995; Tregenza et al., 2000 and this thesis; for other grasshoppers: Chapman et al., 1995; Gibbs and Mousseau, 1994 and other taxa are reviewed in Howard & Blomquist, 2005). A theoretical study by van Doorn et al. (2009) showed that selection favors the evolution of preferences for sexual traits that serve as signals for ecological performance. These signals can then be used to resist matings with nonlocal mates, which will lead to a decrease in gene flow be-

tween locally adapted populations (Klappert & Reinhold 2005; van Doorn *et al.* 2009). In this thesis, I found evidence that CHC signals may contribute to reproductive isolation between *C. biguttulus* and *C. mollis*. Ecological and sexual selection might shape the CHC signals of grasshoppers, because CHC profiles are good indicators of habitat origin or of ecological differences. Two subspecies of *C. parallelus* provide one example for the interaction of ecological selection and sexual selection (Buckley *et al.* 2003). The cuticular blend of these two subspecies significantly correlates with the vegetation of the environment and prior experiments revealed assortative mating of these species between distant locations (Tregenza *et al.* 2000b; Buckley *et al.* 2003). The authors assume that the two subspecies diverged in response to ecological selection, which may incidentally have induced assortative mating between populations (Buckley *et al.* 2003). The differences in food preferences that have been identified in several *Chorthippus* species, including *C. biguttulus* and *C. mollis*, might provide another example of ecological selection interacting with sexual selection (Picaud *et al.* 2003; Berdan *et al.* 2015). This is supported by an F_{st} outlier analysis indicating that several genes that are involved in food preference and metabolic processes in *C. biguttulus* and *C. mollis* were under selection (Berdan *et al.* 2015). Thus, ecological selection may generate variation in chemical phenotypes between populations and lead to assortative mating with sexual selection as the primary selective force leading to speciation.

Species divergence by reinforcement is a further potential scenario for grasshopper speciation in sympatry. In this scenario selection will favor divergence in mating behavior and strengthen prezygotic isolation between species to avoid the production of hybrids. However, speciation by reinforcement requires a previously evolved postzygotic barrier (Butlin 1998; Noor 1999; Seehausen *et al.* 2014). Reinforcement leads to a pattern in which mating signals, signal preferences or both are more divergent in sympatric populations than in allopatric populations. In grasshoppers, assortative mating or differences in mating signals between distinct populations are common, but there is little evidence to date in favor of reinforcement as the mechanism generating these differences (Ritchie *et al.* 1992; Neems & Butlin 1994; Butlin 1998; Tregenza *et al.* 2000b; Reinhardt 2006; Stange 2011). However, it is not straightforward to test for reinforcement in grasshoppers since they often co-occur with many other grasshopper species and community effects may have affected the signal structure and preferences (Römer *et al.*

1989; Amezquita *et al.* 2011; Schmidt *et al.* 2011; Symes 2014). Thus, in such comparisons we need to consider the total biodiversity of grasshoppers at a specific location and not only focus on a specific species pair. Therefore, reinforcement cannot be ruled out as a driver for speciation in *C. biguttulus* and *C. mollis*, but it is challenging to test it in these species.

As an alternative to reinforcement, species divergence may also arise by sexual conflict. Theoretical and empirical work suggests that sexual conflict can drive speciation under specific circumstances in sympatry and allopatry (reviewed in Gavrilets, 2014). In grasshoppers sexual conflict may occur over mating rate. Within the genus *Chorthippus* females of some species are polyandrous, i.e. they typically mate with multiple males (Butlin *et al.* 1987; Reinhardt & Köhler 1999; Wirmer *et al.* 2010). In polyandrous species sperm of different males compete to fertilize the eggs within the female. Traits that lead to an increase in this reproductive competition are favored, even if they convey costs to females (Chapman *et al.* 1995b; Rice 1998). The reproductive system of females will try to lower these costs. This will lead to a coevolutionary arms race between the reproductive systems of males and females, which can then result in divergence of reproductive systems between allopatric populations. As a consequence this divergence can then lead to assortative mating and reproductive isolation between these allopatric populations (Alexander *et al.* 1997; Rice 1998; Gavrilets 2014). In *C. biguttulus* and *C. mollis* one such conflict might occur over copulation time. Longer copulation times might increase reproductive success in males, but not in females. First, males might directly increase their reproductive success by mate guarding (Kirkendall 1984; Alcock 1994; Andrés & Rivera 2000) Second, grasshopper males may decrease female attractiveness to other males, by masking the CHC profile of a female with its own profile. Longer copulation durations may increase the efficiency of masking a females' CHC profile. In *Drosophila melanogaster*, males adjust the copulation duration based on the CHC signal of females. The copulation duration was significantly shorter in crossing when the CHC profile of the unmated female was manipulated by an exchange with a CHC profile of a previously mated female (Friberg 2006).

Analyzing the copulations protocols of interspecific *C. biguttulus* and *C. mollis* crosses (chapter 2) revealed that a copulation duration of 5 minutes was sufficient to transfer the spermatophore and to fertilize the eggs. The number of egg-pods and eggs laid by a female was independent of copulation duration (unpublished data), indicating that females might not benefit

from longer copulation durations. Considering these observations, the mean duration of interspecific matings of chapter 2 was surprisingly long (25 ± 15 min, $N = 44$). The mean (± sd) duration of mating between a *C. biguttulus* female and a *C. mollis* male (19 ± 11 min, N = 14) was significantly lower ($W = 113.5$, $p = 0.015$, Wilcoxon signed-rank test) compared to the reciprocal cross (28 ± 16 min, $N = 30$). One explanation for this result is that copulation durations between species may vary. Alternatively, the larger body size of *C. biguttulus* individuals might enable *C. biguttulus* females to remove the smaller *C. mollis* males and likewise allow *C. biguttulus* males to stay longer on the back of *C. mollis* females. In response to this conflict, selection may have favored longer legs to better grasp the female. Prior research revealed that hindleg length correlates positively with the attractiveness of male songs (Stange & Ronacher 2012). It remains to be tested, if divergence in sexual signals might have evolve as a by-product to sexual conflict (Gavrilets 2014).

Arnqvist et al. (2000) predict sexual conflict as a common evolutionary generator of species diversity in polyandrous insects. These authors showed that the speciation rates in polyandrous insect groups were four times higher than those in related monandrous insect groups (Arnqvist *et al.* 2000). However, other authors remarked that in comparative studies it is difficult to exclude or control for other effects, such as sexual selection (Panhuis *et al.* 2001; Coyne & Orr 2004). Whether selection through sexual conflict can lead to reproductive isolation in grasshoppers requires experiments on the conflicts between males and females and their consequences. Copulation time is only one example of many potential conflicts between sexes. Thus, it is important to assess the conditions under which sexual conflict may promote speciation in grasshoppers.

5.1 Outlook

This thesis has revealed new components of reproductive isolation and mating behavior in the closely related species *C. biguttulus* and *C. mollis*. From an ultimate point of view, it would be of interest to extend my work from chapter 2 and 3 to other species in a comparative framework. Information on the variation of CHC profiles between species, sexes and populations in combination with behavioral tests would increase our understanding of how chemical signals might have contributed to species divergence in grasshoppers.

On a proximate level, the bioassay described in chapter 4 is very suitable to test various aspects of chemical signaling in grasshoppers and to test if male mate choice is present in this system. It would be particularly interesting to see how specific factors, like local adaptation, food preferences, age or mating status affect the CHC composition and thus behavior. Further, my work may inspire future research to examine the role of multimodal signals in courtship behavior (Candolin 2003; Hebets & Papaj 2005; Mérot *et al.* 2015) and the interaction of acoustic and chemical signals on mating decision (Simmons *et al.* 2013).

On a molecular level, the candidate genes described in chapter 3 may encourage further studies to use genetic tools, like the CRISPR-Cas9 system or RNA interference, to investigate the molecular basis of CHC production and chemosensory behavior. From a broader perspective this thesis underlined the complexity of isolation mechanisms and provides new insights into reproductive behavior in grasshoppers. Additional studies on the molecular basis of reproductive isolation barriers may open the possibility to validate and discard certain speciation scenarios in grasshoppers.

Appendix

A Chapter 2

Table A.1 Pairwise comparisons for hatching success & survival rate of larvae
Columns for hatching success give results from Tukey post hoc test, with p values adjusted for multiple comparisons using the Shaffer method. The column for Survival rate of larvae gives results Chi square post hoc test, with p values adjusted for multiple comparisons using the FDR method. Significant values are indicated in bold.

	hatching success			Survival rate of larvae
Comparison	SE	*Z*	*P*	*P*
C. biguttulus x *C. mollis*	0.401	-1.817	0.138	**0.002**
C. biguttulus x BIMO	0.313	-7.35	**<0.001**	**<0.001**
C. biguttulus x MOBI	0.291	-7.324	**<0.001**	**<0.001**
C. biguttulus x Backcross	0.404	2.901	**0.015**	**<0.001**
C. mollis x BIMO	0.357	4.401	**<0.001**	0.278
C. mollis x MOBI	0.338	-4.152	**<0.001**	0.180
C. mollis x Backcross	0.439	1.011	0.624	0.278
Backcross x BIMO	0.361	-3.126	**0.007**	1
Backcross x MOBI	0.342	-2.807	**0.015**	1
BIMO x MOBI	0.227	0.738	0.624	1

Table A.2 The strength of reproductive isolation barriers for the Backcrosses generation.
RIs are calculated relative to the measurements of F1 hybrids as the 'parental species'. I defined the measurements of the Backcrosses as the 'heterospecific' value and the measurements of the F1 hybrids as the 'conspecific' value in the equation 4A. See methods for details.

	C. biguttulus[1] x *C. mollis*[2]			*C. mollis*[1] x *C. biguttulus*[2]		
Reproductive isolation barriers	RI value	cumulative strength	absolute contribution	RI value	cumulative strength	absolute contribution
prezygotic barriers						
calling song preference	0.917	0.9174	0.917	0.7540	0.7540	0.754
chemical cues short range (male choosiness)	0.486	0.9706	0.053	0.2308	0.8388	0.085
acoustic signal short range (mating success)	0.12	0.9768	0.006	NA	NA	NA
{mating success no choice experiment (chemical cues)	*1*	*1.0000*	*0.023*	*1*	*1.0000*	*0.1612}*
postzygotic barriers	F1 hybrids x *C. biguttulus*					
hatching success of fertilized eggs	-0.06	0.9739	-0.003			
survival rate of larvae	0.138	0.9802	0.006			
functional development of wing and hind leg morphology	0.017	0.9808	0.001			
courtship motivation of females	-0.63	0.9183	-0.063			
Total isolation		**0.9183**				

1 conspecific species

2 heterospecific species

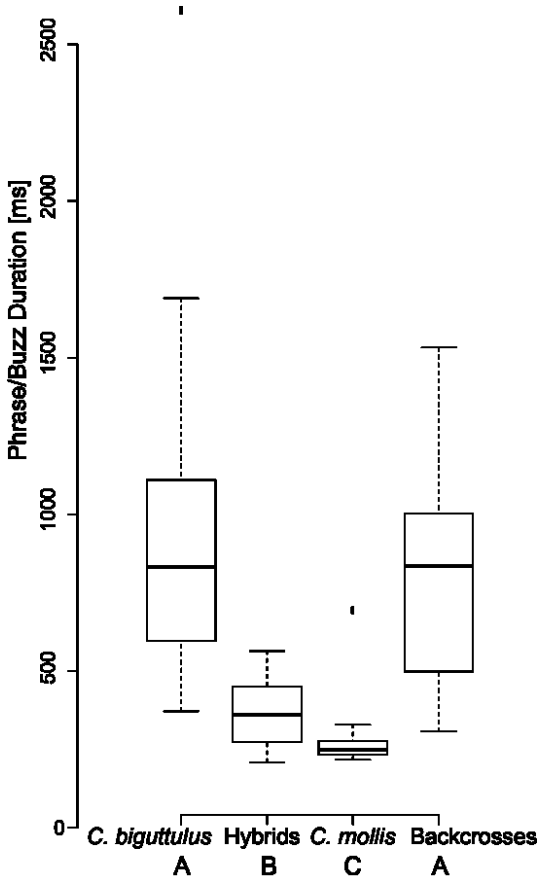

Figure A.1 Duration of buzzes/phrases of female songs.
The duration of buzzes and phrases was averaged across all responses of one female and across all females of one species group. In total the mean buzz/phrase duration of 32 *C. biguttulus*, 25 *C. mollis*, 27 F1 hybrid and 8 Backcross females was taken. Capitals below the species groups represent significance in phrase/buzz duration between test groups (Kruskal-Wallis test $p < 0.01$).

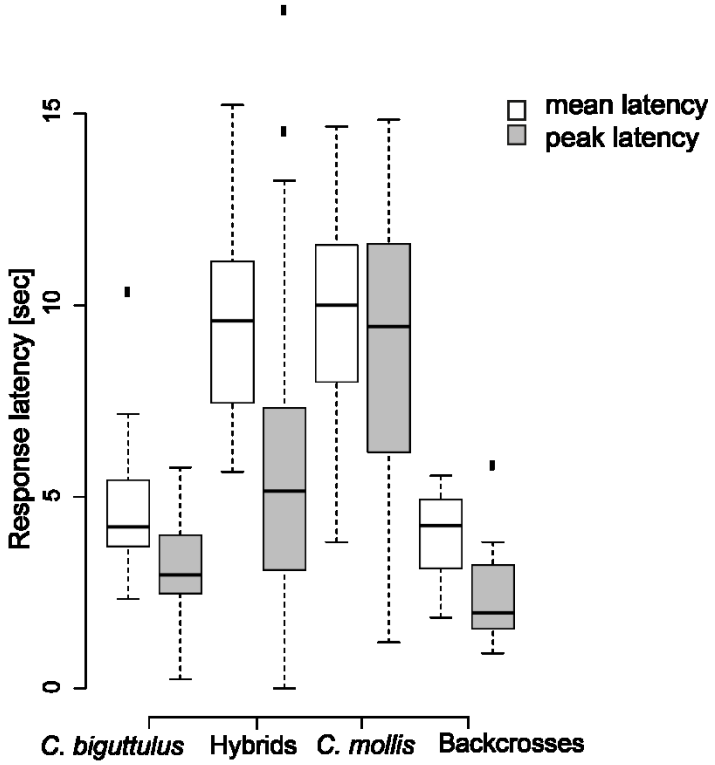

Figure A.2 Response latency of female songs.
The mean response latency was averaged across all responses of one female and across all females of one species group (blank boxes). To estimate the peak latency I averaged the latency of all responses to the peak preference of one female and averaged afterwards the response latencies of the peak preference across all females of a species group (filled boxes). I measured the mean and the peak response latencies from 32 *C. biguttulus*, 32 *C. mollis*, 32 F1 hybrid and 9 Backcross females.

Table A.3 Strength of postzygotic reproductive isolation barriers.

Reproductive isolation barriers	*C. biguttulus*[1] x *C. mollis*[2]			*C. mollis*[1] x *C. biguttulus*[2]		
	RI value	cumulative strength	absolute contribution	RI value	cumulative strength	absolute contribution
postzygotic barriers						
hatching success of fertilized eggs	0.292	0.2918	0.292	0.242	0.2416	0.242
survival rate of larvae	0.096	0.3769	0.085	-0.039	0.2044	-0.037
functional development of wing & hind leg morphology	0.099	0.4587	0.082	0.084	0.2836	0.079
courtship motivation of females	0.607	0.8338	0.375	0.621	0.7692	0.486
behavioral isolation of F1 hybrid males (acoustic)	0.666	0.9643	0.13	0.696	0.9543	0.185
behavioral isolation F1 hybrid females (acoustic)	0.727	0.9943	0.030	0.727	0.9926	0.038
hatching success of fertilized backcross eggs	0.237	0.9965	0.002	0.185	0.9949	0.002
survival rate of backcrosses of larvae	0.231	0.9978	0.001	0.121	0.9960	0.001
functional development of wing & hind leg morphology BC	0.115	0.9982	4.59E-04	0.101	0.9967	7.3E-04
courtship motivation of backcross females	0.017	0.9983	5.82E-05	0.000	0.9967	-1.2E-07
behavioral isolation backcross females	0.06	0.9985	1.9E-04	0.478	0.9988	0.002
Total isolation		**0.9985**			**0.9988**	

1 conspecific species

2 heterospecific species

Table A.4 Statistic results of F1 hybrid male song characteristics on female response.

		tick structure						syllable structure						buzz duration					
		Species group		Song variant		Species group x Song variant		Species group		Song variant		Species group x Song variant		Species group		Song variant		Species group x Song variant	
Comparison	*N*	$\chi 1^2$	*P*	$\chi 3^2$	*P*	$\chi 3^2$	*P*	$\chi 1^2$	*P*	$\chi 6^2$	*P*	$\chi 6^2$	*P*	$\chi 1^2$	*P*	$\chi 9^2$	*P*	$\chi 9^2$	*P*
C. biguttulus x *C. mollis*	13 15	61.8	<0.001	5	0.17	2.9	0.41	22	<0.001	439	<0.001	211	<0.001	29.2	<0.001	90.5	<0.001	68.4	<0.001
C. biguttulus x Backcross	13 9	12	<0.001	1	0.852	8643	<0.001	3	0.085	407	<0.001	47	<0.001	11	0.001	246	<0.001	7218	<0.001
C. mollis x Backcross	15 9	13.42	<0.001	13.71	0.003	1.27	0.737	1.8	0.175	15.5	0.017	55.6	<0.001	9	0.003	826	<0.001	81	<0.001

Table A.5 Statistic results on the likelihood of female response based on Figure 2.5

	pause duration between buzzes							variation syllable duration							pause duration between syllables						
		Species group		Song variant		Species group x Song variant			Species group		Song variant		Species group x Song variant			Species group		Song variant		Species group x Song variant	
Comparison	*N*	$\chi1^2$	*P*	$\chi1^2$	*P*	$\chi1^2$	*P*	*N*	$\chi1^2$	*P*	$\chi1^2$	*P*	$\chi1^2$	*P*	*N*	$\chi1^2$	*P*	$\chi1^2$	*P*	$\chi1^2$	*P*
C. biguttulus x *C. mollis*	37 / 38	31.4	<0.001	29.2	<0.001	288.7	<0.001	45 / 20	38.4	<0.001	13.4	<0.001	8.1	0.004	46 / 37	61.3	<0.001	1.5	0.22	0.5	0.48
C. biguttulus x F1 hybrid	37 / 31	0	0.949	23.74	<0.001	3.46	0.063	45 / 31	82.8	<0.001	14.7	<0.001	5.6	0.018	46 / 31	110.8	<0.001	3.2	0.076	1	0.314
C. biguttulus x Backcross	37 / 8	1	0.295	14175	<0.001	3	0.097	45 / 8	7.15	0.008	22.94	<0.001	0.01	0.939	46 / 8	5.42	0.02	1.86	0.17	0.1	0.76
C. mollis x F1 hybrid	38 / 31	22.79	<0.001	31.32	<0.001	9.07	0.003	20 / 31	0.17	0.68	0.2	0.66	0.51	0.47	37 / 31	1.52	0.217	2.98	0.085	2.63	0.105
C. mollis x Backcross	38 / 8	17.15	<0.001	9.68	0.002	5.18	0.023	20 / 8	6.45	0.011	1.75	0.186	9	0.003	37 / 8	8.03	0.005	0.38	0.538	0.23	0.63
Backcross x *F1 hybrid*	8 / 31	1.64	0.2	21.42	<0.001	17.62	<0.001	8 / 31	11.14	<0.001	3.23	0.072	6.53	0.011	8 / 31	14.54	<0.001	5.96	0.015	1.87	0.171
BIMO x MOBI	12 / 19	0.47	0.49	19.38	<0.001	2.17	0.14	12 / 19	1.61	0.2	0.01	0.93	6.58	0.01	12 / 19	0.53	0.466	5.75	0.016	2.93	0.087

B Chapter 3

Table B.1 Identification & ortholog assignment of FAS and elongases in Chorthippus

Taxon	Accessionnumber	Ensemble identifier & gene name
Fatty acid synthases		
Chorthippus biguttulus		20011big_P1-comp52607_c0_seq1
		20011big_P1-comp58522_c0_seq1
		20013big_P1_male-comp38343_c0_seq2
		20030big_male-comp17321_c0_seq1
		20030big_male-comp37496_c1_seq1
Chorthippus mollis		20003mol_P1-comp70825_c0_seq1
		20003mol_P1-comp71027_c0_seq1
		20003mol_P1-comp71695_c0_seq1
		20016mol_P1_male-comp81435_c0_seq1
		20164mol-comp17321_c0_seq1
Drosophila melanogaster	AAF51148.1	CG3523
	AAF51149.1	CG3524
	EAA46042.3	CG17374
Elongases		
Chorthippus biguttulus		20001big_P1-comp67133_c0_seq1
		20008big_male-comp94799_c0_seq1
		20008big_male-comp98995_c0_seq1
		20010big_P1-comp55033_c0_seq1
		20013big_P1_male-comp131546_c0_seq1
		20013big_P1_male-comp77836_c1_seq1
		20030big_male-comp106526_c0_seq1
		20030big_male-comp88504_c2_seq1
		20030big_male-comp89598_c0_seq1
		20030big_male-comp90320_c0_seq1
		20030big_male-comp91260_c0_seq1
		20030big_male-comp94699_c0_seq1
Chorthippus mollis		20007mol_male-comp111352_c0_seq1
		20007mol_male-comp113584_c0_seq1
		20015mol_P1_male-comp119420_c0_seq1
		20015mol_P1_male-comp86102_c0_seq1
		20016mol_P1_male-comp83867_c0_seq1
		20056mol-comp120587_c6_seq3
		20164mol-comp17390_c0_seq1
		20164mol-comp39997_c0_seq1

		20164mol-comp41288_c0_seq1
		20164mol-comp42127_c0_seq1
		20164mol-comp45532_c0_seq1
Drosophila melano-gaster	NP_001097580.1	CG11801 (Elo68beta)
	AAF54462.1	CG16904
	AAF54461.1	CG16905
	AAF57646.3	CG17821
	AAF57647.2	CG18609
	NP_649754.1	CG2781
	AAM71039.1	CG30008
	AAN13958.2	CG31141
	NP_730843.2	CG31522
	NP_649474.1	CG31523
	NP_729666.2	CG32072 (Elo68alpha)
	AAF56020.2	CG33110
	NP_648909.1	CG3971 (baldspot)
	NP_651063.1	CG5278
	AAF56015.1	CG5326
	NP_732761.1	CG6921 (james bond)
	AAF54460.1	CG8534
	AAF54464.1	CG9458
	AAF54463.2	CG9459

Table B.2 Mean (±sd) relative composition [%] of the CHC profiles of *C. biguttulus* & *C. mollis*

Peak	Retention time [min]	Compound	*C. mollis* Females *N* = 17	*C. mollis* Males *N* =34	*C. biguttulus* Females *N* = 40	*C. biguttulus* Males *N* = 34
1	17.60	*n*-C25	2.6±1.4	1.7±1.0	5.3±2.8	4.1±3.0
2	19.10	*n*-C27	12.2±4.3	13.3±4.3	8.6±3.7	8.4±3.7
3	20.51	*n*-C29	26.4±6.3	19.1±3.2	21.3±3.8	18.0±4.0
4	21.00	3-MeC29	0.1±0.3	-	0.3±0.3	0.1±0.2
5	21.80	*n*-C31	10.0±2.4	6.5±1.3	8.5±2.0	7.5±2.4
6	22.00	13-MeC31	-	*tr*	0.2±0.3	0.2±0.2
7	22.45	*n*-C32	*tr*	*tr*	*tr*	-
8	23.14	*n*-C33	1.3±0.4	0.9±0.3	0.8±0.5	0.9±0.5
9	23.37	11-/13-/15-MeC33	*tr*	0.3±0.3	1.7±1.2	1.4±0.8
10	23.50	unidentified	-	-	-	0.8±0.8
11	23.54	15,19-/13,x-/11,x-diMeC33	3.3±1.6	4.7±1.0	0.2±0.7	0.2±1.1
12	23.60	13,19-/11,21-/9,x-diMeC33	0.2±0.8		4.0±3.7	1.8±1.5
13	23.82	13,x,x-/11,x,x-/9,x,x-diMeC33	2.2±1.0	2.2±0.5	0.6±1.4	0.2±0.7
14	24.19	10-/11-/12-/13-/14-MeC34	-	-	0.2±0.4	0.1±0.3
15	24.42	11,x-/12,x-/13,x-/14,x-diMeC34	0.6±0.6	0.7±0.3	*tr*	*tr*
16	25.09	11-/13-/15-/17-MeC35	1.4±0.7	2.3±0.7	5.7±1.9	5.6±1.9
17	25.34	15,19-/13,17-/11,15-diMeC35	13.2±5.1	22.8±2.9	1.7±5.2	1.4±5.8
18	25.40	13,x-/11,23-/9,x-diMeC35	-	-	8.3±11.0	24.9±11.5
19	25.47	11,x-/9,x-/7,x-diMeC35	0.5±2.1	-	9.0±8.8	1.1±3.7
20	25.58	15,19,x-/13,17,x-triMeC35	11.8±4.5	12.5±2.0	0.8±2.6	0.7±2.9
21	25.61	13,17,x-/11,15,x-triMeC35	-	-	1.9±2.8	5.0±2.5
22	25.64	11,x,x-/9,x,x-diMeC35	0.2±0.9	-	2.0±2.5	0.2±0.6
23	25.89	3,x-diMeC35	1.1±1.8	0.1±0.3	0.2±0.6	-
24	26.47	12-/13-/14-/15-/16-MeC36	0.4±0.6	1.3±0.4	1.2±0.7	1.5±0.6
25	26.71	unidentified	0.6±0.6	0.8±0.4	*tr*	*tr*
26	27.38	11-/13-/15-/17-/19-MeC37	1.2±0.3	1.2±0.4	2.7±1.0	2.5±0.9
27	27.71	15,19-/15,21-/13,23-diMeC37	4.0±1.7	6.0±1.5	0.7±2.2	0.4±1.8
28	27.76	13,x- (17/23)/11,x-(25)/9,x-diMeC37	0.2±1.0	-	8.0±3.0	8.2±3.4
29	28.01	15,19,x-/13,17,x-triMeC37	4.1±2.0	3.3±1.0	0.4±1.1	0.2±0.9
30	28.05	13,17,x-/11,x,x-/9,x,x-triMeC37	-	-	0.9±1.4	1.7±0.9
31	28.13	11,15,x-/9,13,x-diMeC37	-	-	0.3±0.8	0.3±1.1
32	30.54	i-MeC39	-	-	*tr*	0.1±0.2
33	30.98	15,x-/13,x-diMeC39	*tr*	-	0.1±0.3	0.2±0.5
34	31.05	11,x-(27)/9,x-diMeC39	-	-	0.8±1.2	0.1±0.4
		Number of compounds	16.1±1.9	16.9±1.1	16.7±1.8	16.9±1.6
		n-alkanes	52.7±9.9	41.6±7.1	44.6±7.6	39.0±10.1
		methyl-branched alkanes	3.0±1.2	5.1±1.5	11.5±3.8	11.3±3.7
		dimethyl-branched alkanes	22.1±6.1	34.3±4.5	32.8±5.5	38.4±8.5
		trimethyl-branched alkanes	18.3±6.4	17.9±2.9	6.5±4.0	8.0±3.6
		others	1.0±0.7	0.9±0.4	3.4±2.0	2.8±1.4

tr traces (<0.1%)

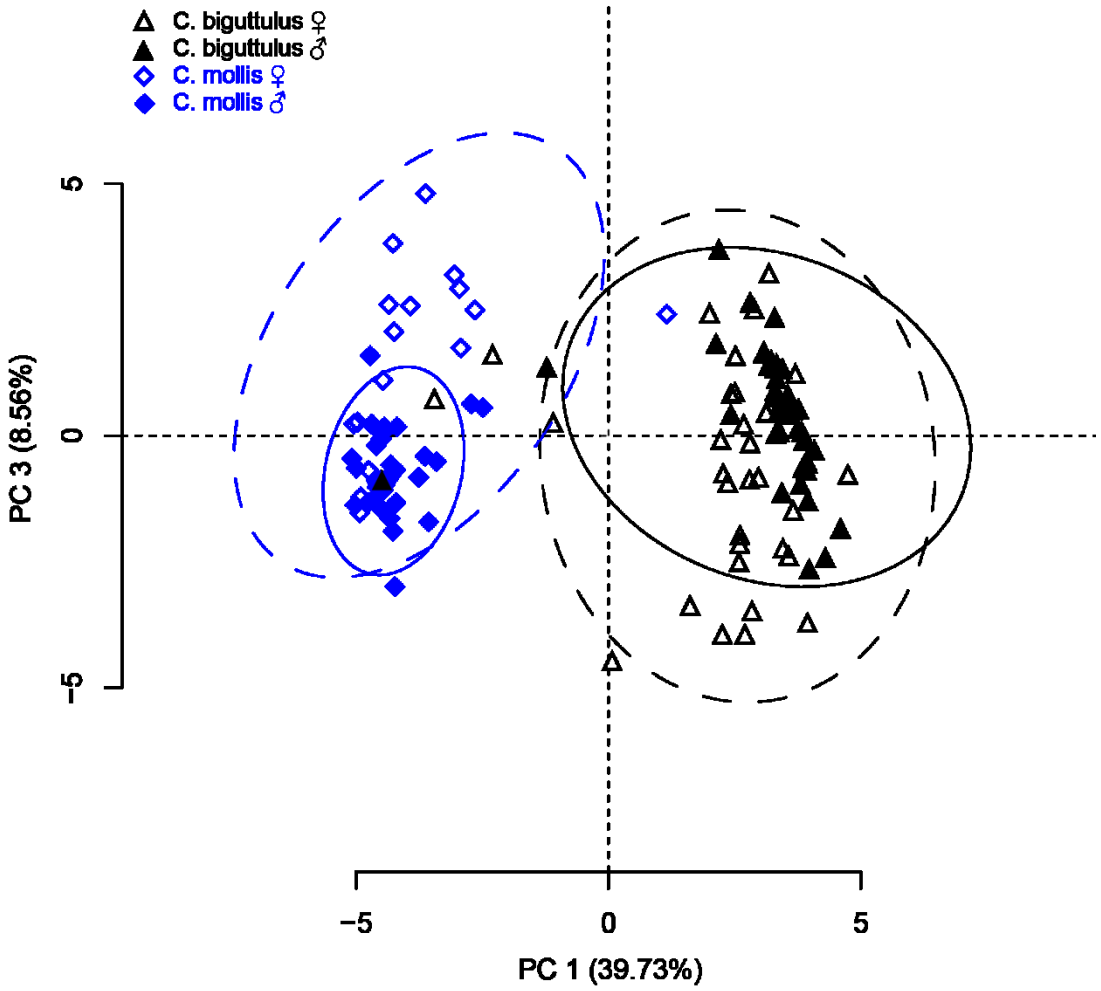

Figure B.1 Principal component analysis (PCA) of CHC phenotypes.
Shown are principal component (PC) 1 *versus* 3 with variances explained by each PC given in parentheses. Ellipses indicate 95% confidence intervals. The PCA is based on the relative composition of 34 CHC peaks (see Table 3.1 for loadings).

Table B.3 Calculation of substitution rates of FAS and ELO candidate genes

Family	Contig name in reference transcriptome	Length[a]	Substitutions			dN/dS	*P*
	C. biguttulus		N	S	total		
FAS	20030big_male-comp37496_c1_seq1	7365	17	64	81	0.109	<0.0001
FAS	20013big_P1_male-comp38343_c0_seq2	6936	1	3	4	0.103	0.0413
FAS	20011big_P1-comp52607_c0_seq1	1149	3	0	3	-	-
FAS	20030big_male-comp38169_c0_seq1	6531	16	39	55	0.102	<0.0001
Elo	20010big_P1-comp55033_c0_seq1	870	-	2	2	-	-
Elo	20013big_P1_male-comp131546_c0_seq1	870	0	0	0	-	-
Elo	20030big_male-comp106526_c0_seq1	747	0	0	0	-	-
Elo	20008big_male-comp98995_c0_seq1	1209	1	2	3	0.129	0.1038
Elo	20013big_P1_male-comp77836_c1_seq1	810	2	9	11	0.003	<0.0001
Elo	20030big_male-comp89598_c0_seq1	795	1	6	7	0.061	0.002
Elo	20030big_male-comp88504_c2_seq1	948	1	12	13	0.028	0.0668
Elo	20030big_male-comp94699_c0_seq1	303	3	0	3	-	-
Elo	20030big_male-comp91260_c0_seq1	1005	1	5	6	0.046	0.0011
Elo	20008big_male-comp94799_c0_seq1	963	0	2	2	0	-
Elo	20030big_male-comp90320_c0_seq1	954	0	5	5	0	-

[a] Length of the open reading frame of *C. biguttulus.*

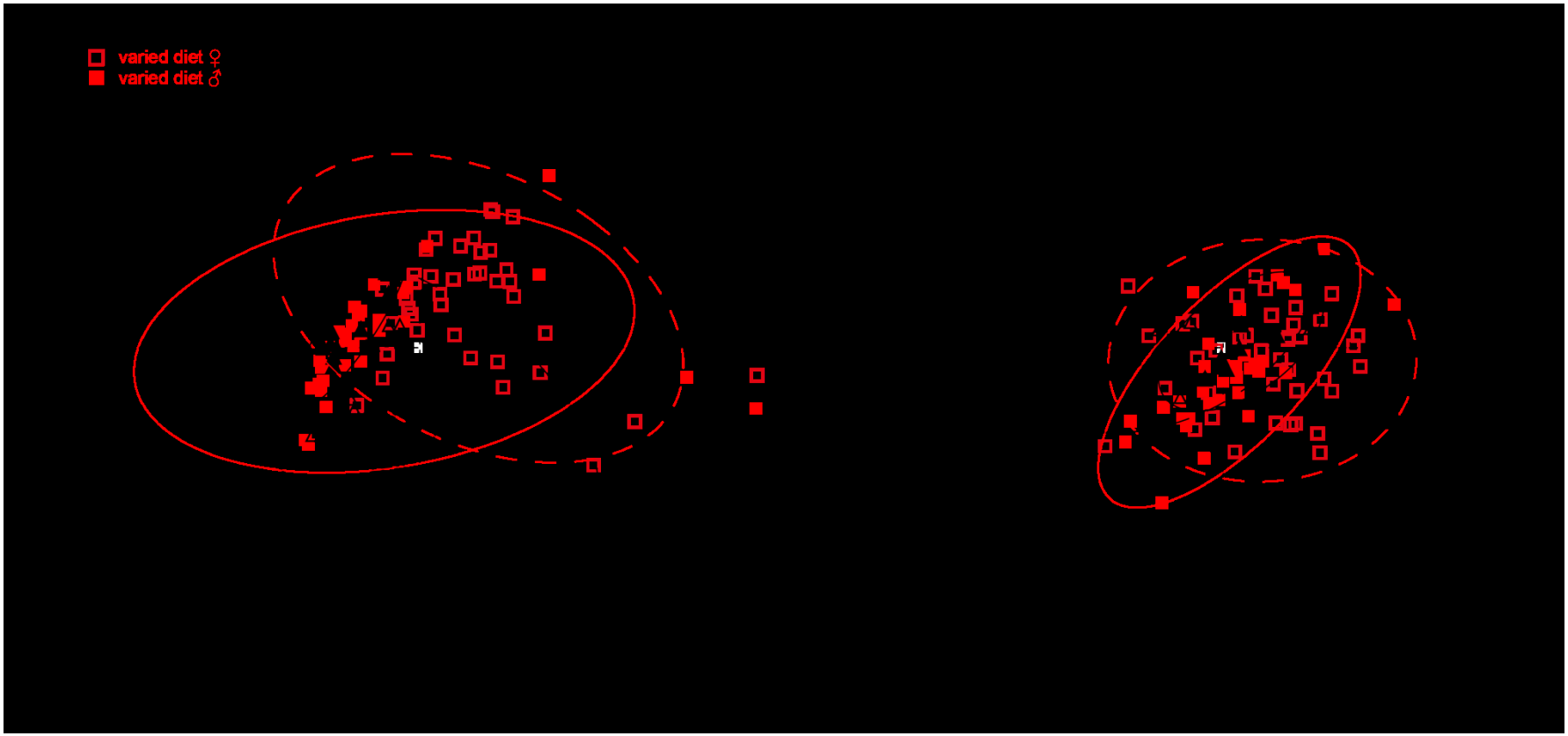

Figure B.2 The effect of diet variation on the CHC profile in *C. biguttulus*.
Shown are principal component (PC) 1 *versus* 3 (A) and 3 versus 4 (B) with variances explained by each PC given in parentheses. Ellipses indicate 95% confidence intervals. The principal component analysis is based on the relative composition of 40 CHC peaks (see Table B.6, B.7 for details)

Table B.4 Statistics of the CHC variation caused by diet variation in *C. biguttulus*.

Linear models were used to test for variation in diet effects as explanatory variable on the pc scores of pc1-5 as the dependent variable. Shown are the results for pc 1-4 (model for pc5 showed no significands). Tukey's HSD post hoc tests were used for pairwise comparisons of treatment groups. Significant differences are labeled in bold digits. Total N = 114

	PC1		PC2		PC3		PC4	
Variation in diet	$F_{3,110}$	*P*	$F_{3,110}$	*P*	$F_{3,110}$	*P*	$F_{3,110}$	*P*
F-statistic linear Model	10.6	**<0.001**	12.5	**<0.001**	17.9	**<0.001**	10.4	**0.002**
Comparison *varied diet vs simple diet*	Coefficients *(SE)*		Coefficients *(SE)*		Coefficients *(SE)*		Coefficients *(SE)*	
Treatment *(varied vs simple)*	2.11 *(0.58)*	**<0.001**	0.25 *(0.52)*	0.638	2.89 *(0.4)*	**<0.001**	-1.54 *(0.34)*	**<0.001**
Sex	-1.58 *(0.92)*	0.090	2.87 *(0.83)*	**<0.001**	*2.29 (0.63)*	**<0.001**	-1.00 *(0.54)*	0.070
Treatment x Sex	-1.09 *(1.08)*	0.316	-0.43 *(0.97)*	0.661	-3.21 *(0.75)*	**<0.001**	0.67 *(0.64)*	0.295
post-hoc Tukey's HSD tests		*P adj*		*P adj*		*P adj*		*P adj*
simple diet ♀ x varied diet ♀		**0.002**		0.965		**<0.001**		**<0.001**
simple diet ♀ x varied diet ♂		0.796		**<0.001**		**<0.001**		**<0.001**
simple diet ♂ x varied diet ♀		**<0.001**		0.008		0.761		0.736
simple diet ♂ x varied diet ♂		0.676		0.996		0.955		0.379
simple diet ♂ x simple diet ♀		0.322		0.004		**0.003**		0.265
varied diet ♂ x varied diet ♀		**<0.001**		**<0.001**		0.088		0.771

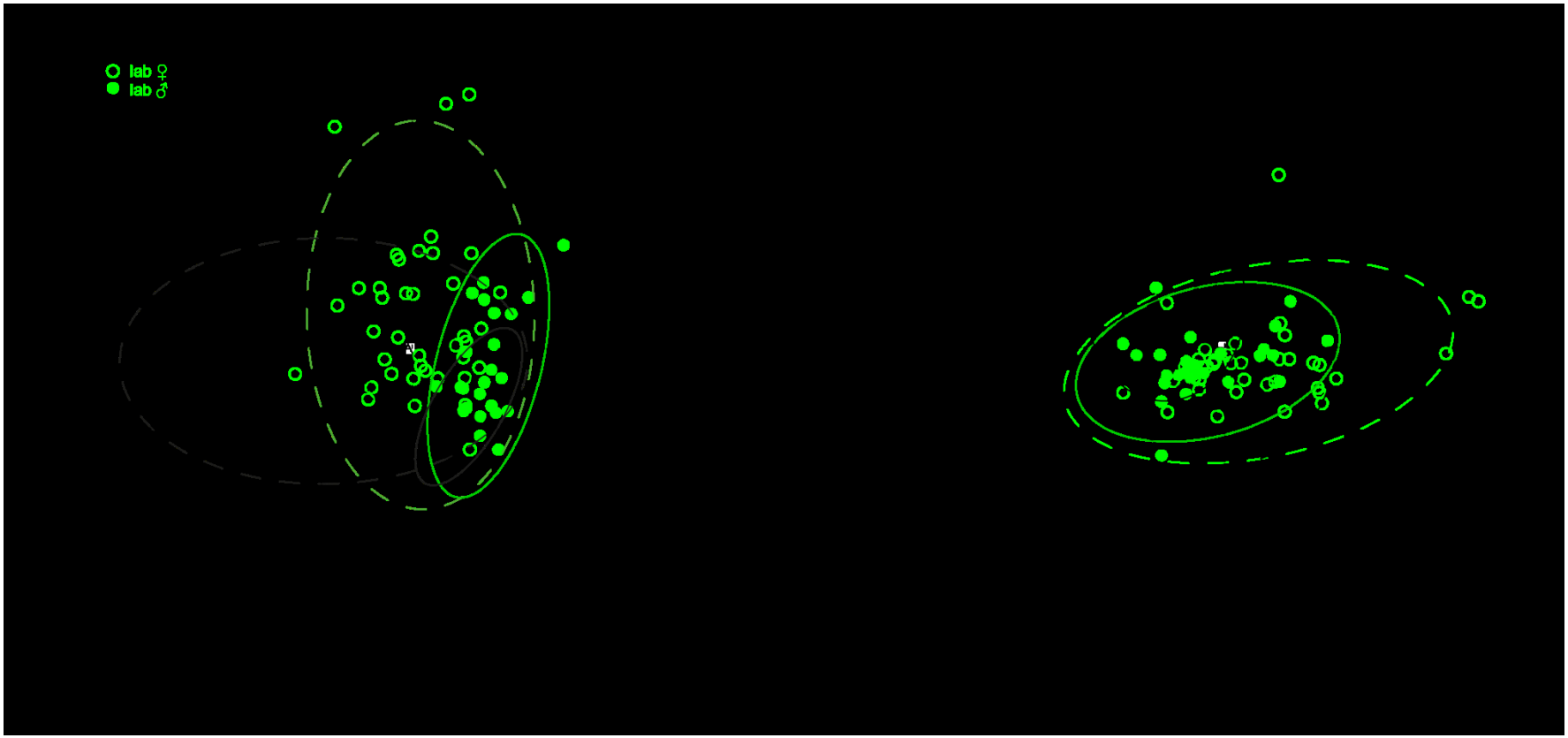

Figure B.3 The effect of rearing conditions on the CHC profile in *C. biguttulus*
Shown are principal component (PC) 2 *versus* 3 (A) and 3 *versus* 5 (B) with variances explained by each PC given in parentheses. Ellipses indicate 95% confidence intervals. The principal component analysis is based on the relative composition of 40 CHC peaks (see Table B.6, B.7 for details)

Table B.5 Statistics of the CHC variation caused by rearing differences in *C. biguttulus*.

Linear models were used to test for rearing effects as explanatory variable on the pc scores of pc1-5 as the dependent variable. Shown are the results for pc 2, 3, 5 (models for pc1, 4 showed no significands). Tukey's HSD post hoc tests were used for pairwise comparisons of treatment groups. Significant differences are labeled in bold digits. Total N = 104.

	PC2		PC3		PC5	
Rearing condition	$F_{3,100}$	*P*	$F_{3,100}$	*P*	$F_{3,100}$	*P*
F-statistic linear Model	45.5	**<0.001**	8.8	**<0.001**	6.5	**<0.001**
Comparison *field vs lab*	Coefficients *(SE)*		Coefficients *(SE)*		Coefficients *(SE)*	
Treatment *(field or lab)*	2.67 *(0.39)*	**<0.001**	1.47 *(0.43)*	**<0.001**	-0.91 *(0.31)*	**0.004**
Sex	4.12 *(0.6)*	**<0.001**	*-1.29 (0.66)*	0.054	0.68 *(0.48)*	0.156
Treatment x Sex	-2.21 *(0.72)*	**0.003**	-0.35 *(0.8)*	0.664	-0.70 *(0.57)*	0.224
post-hoc Tukey's HSD tests		*P adj*		*P adj*		*P adj*
field ♀ *x lab* ♀		**<0.001**		**0.005**		**0.022**
field ♀ *x lab* ♂		**<0.001**		0.981		**0.031**
field ♂ *x lab* ♀		0.071		**<0.001**		**0.006**
field ♂ *x lab* ♂		0.872		0.347		**0.007**
field ♂ *x field* ♀		**<0.001**		0.214		0.484
lab ♂ *x lab* ♀		**<0.001**		0.347		1.000

Table B.6 Factor loadings for both treatments (rearing condition and diet variation)

			PC1		PC2		PC3		PC4		PC5	
Peak	Retention Time	Compound	Rearing	Diet	Rearing	Diet	Rearing	Diet	Rearing	Diet	Rearing	Diet
1	17.60	*n*-C25	0.02	0.08	0.06	-0.10	0.34	0.22	-0.10	0.26	0.10	0.24
2	19.10	*n*-C27	0.02	0.07	0.15	0.05	0.21	0.28	0.13	-0.01	-0.20	0.19
3	20.51	*n*-C29	-0.01	0.13	0.16	0.04	0.29	0.31	0.22	0.22	-0.01	0.04
4	21.00	3-MeC29	0.14	0.15	-0.09	-0.11	0.14	-0.19	-0.23	0.28	-0.14	0.02
5	21.15	*n*-C30	-0.06	-0.08	0.06	0.07	-0.14	0.03	-0.15	0.08	0.26	-0.05
6	21.80	*n*-C31	-0.02	0.05	0.26	0.10	0.21	0.34	0.21	0.28	0.20	-0.04
7	22.00	13-MeC31	-0.02	-0.14	-0.11	-0.02	-0.16	-0.16	-0.09	-0.02	0.07	0.16
8	22.21	Cholesterol	-0.01	-0.04	-0.12	-0.20	0.20	0.00	-0.14	0.26	0.19	0.13
9	22.29	3-MeC31	0.10	0.07	-0.08	-0.02	0.11	-0.16	-0.18	0.24	0.00	0.18
10	22.45	*n*-C32	0.03	-0.04	-0.04	0.02	-0.02	-0.02	-0.02	0.35	0.61	0.00
11	23.14	*n*-C33	-0.01	0.00	0.17	0.14	0.02	0.17	0.20	0.20	0.18	-0.15
12	23.37	11-/13-/15-MeC33	-0.08	-0.17	-0.06	-0.11	-0.30	0.10	0.17	-0.16	-0.02	0.36
13	23.50	unidentified	-0.09	-0.15	0.17	0.19	-0.17	0.11	0.26	-0.26	0.02	-0.03
14	23.54	15,19-/13,x-/11,x-diMeC33	0.31	0.25	0.08	0.19	-0.08	-0.10	0.03	-0.07	-0.14	0.06
15	23.60	13,19-/11,21-/9,x-diMeC33	-0.18	-0.09	-0.15	-0.24	0.13	0.09	0.02	-0.17	0.06	-0.06
16	23.75	unidentified	0.16	0.12	0.02	-0.02	-0.06	0.01	0.16	-0.08	-0.07	0.13
17	23.82	13,x,x-/11,x,x-/9,x,x-diMeC33	-0.02	0.10	-0.16	-0.16	0.27	-0.05	-0.11	-0.04	-0.05	-0.34
18	24.19	10-/11-/12-/13-/14-MeC34	0.00	-0.01	-0.20	-0.12	-0.04	-0.14	-0.01	-0.14	0.06	0.16
19	24.44	unidentified	-0.07	0.03	0.17	0.16	0.11	0.38	0.02	0.00	-0.34	0.07
20	24.42	11,x-/12,x-/13,x-/14,x-diMeC34	-0.03	0.15	0.30	0.16	0.18	0.13	0.17	-0.20	0.02	-0.17
21	25.09	11-/13-/15-/17-MeC35	-0.12	-0.12	-0.03	-0.11	-0.23	0.11	0.17	-0.22	-0.12	0.40
22	25.27	unidentified	0.01	-0.19	0.26	0.16	-0.19	-0.12	0.12	-0.05	0.04	0.03
23	25.34	15,19-/13,17-/11,15-diMeC35	0.33	0.27	0.05	0.02	-0.09	0.11	0.01	-0.09	-0.12	0.04

24	25.40	13,x-/11,23-/9,x-diMeC35	-0.28	-0.24	0.14	0.22	0.06	-0.08	-0.07	0.14	0.00	-0.04
25	25.47	11,x-/9,x-/7,x-diMeC35	0.08	0.15	-0.09	-0.25	0.00	0.21	0.22	-0.18	0.16	0.01
26	25.58	15,19,x-/13,17,x-triMeC35	0.34	0.29	0.05	0.19	-0.06	-0.14	0.00	-0.05	0.00	0.08
27	25.61	13,17,x-/11,15,x-triMeC35	-0.17	-0.26	0.27	0.23	-0.07	0.02	-0.27	0.09	0.01	-0.01
28	25.64	11,x,x-/9,x,x-diMeC35	-0.02	0.13	-0.26	-0.31	0.12	0.11	0.35	-0.10	-0.01	-0.13
29	25.89	3,x-diMeC35	0.04	0.11	-0.12	-0.11	0.25	-0.14	-0.11	0.17	-0.17	-0.02
30	26.47	12-/13-/14-/15-/16-MeC36	-0.07	-0.12	-0.01	0.06	-0.20	-0.10	-0.17	-0.19	-0.23	-0.12
31	26.71	unidentified	0.09	0.13	0.15	0.19	0.03	0.08	0.01	-0.12	-0.10	-0.30
32	27.38	11-/13-/15-/17-/19-MeC37	-0.07	-0.05	-0.15	-0.14	-0.24	-0.04	0.10	0.03	-0.12	0.11
33	27.71	15,19-/15,21-/13,23-diMeC37	0.34	0.29	0.04	0.18	-0.06	-0.15	0.00	-0.03	0.01	0.11
34	27.76	13,x- (17/23)/11,x-(25)/9,x-diMeC37	-0.31	-0.29	0.02	-0.10	-0.05	0.12	0.07	-0.03	-0.02	-0.12
35	28.01	15,19,x-/13,17,x-triMeC37	0.34	0.29	0.05	0.19	-0.06	-0.14	0.00	-0.04	0.02	0.11
36	28.05	13,17,x-/11,x,x-/9,x,x-triMeC37	-0.16	-0.25	0.22	0.22	-0.13	0.01	-0.25	0.10	0.06	-0.07
37	28.13	11,15,x-/9,13,x-diMeC37	-0.01	0.10	-0.23	-0.23	0.11	0.02	0.32	0.06	0.01	-0.15
38	30.54	i-MeC39	-0.03	-0.06	-0.29	-0.13	-0.06	-0.26	-0.09	0.08	0.02	-0.05
39	30.98	15,x-/13,x-diMeC39	0.25	0.02	0.04	0.12	-0.05	-0.14	-0.06	-0.03	0.24	0.24
40	31.05	11,x-(27)/9,x-diMeC39	-0.10	0.01	-0.23	-0.23	-0.06	-0.07	0.11	0.04	-0.03	-0.20

Table B.7 The effect of environmental conditions on the relative composition [%] of the CHC profiles in *C. biguttulus*

Peak	Retention time (min)	Compound	*C. biguttulus*					
			Field - variet diet		Field - simple diet		Lab - simple diet	
			Females *N* = 40	Males *N* = 34	Females *N* = 31	Males *N* = 9	Females *N* = 36	Males *N* = 28
1	17.60	*n*-C25	5.3±2.8	4.1±3.0	5.6±3.0	4.0±1.4	6.0±4.3	4.8±2.7
2	19.10	*n*-C27	8.6±3.7	8.4±3.7	7.2±2.5	7.1±3.0	8.5±3.3	9.2±3.7
3	20.51	*n*-C29	21.3±3.8	18.0±4.0	20.9±3.8	18.5±3.1	21.9±3.7	20.5±4.1
4	21.00	3-MeC29	0.3±0.3	0.1±0.2	0.6±0.4	0.2±0.2	0.5±0.5	0.3±0.4
5	21.15	*n*-C30	-	-	0.1±0.4	0.6±0.7	0.1±0.2	-
6	21.80	*n*-C31	8.5±2.0	7.5±2.4	8.5±2.7	8.7±2.4	10.2±2.2	9.8±2.5
7	22.00	13-MeC31	0.2±0.3	0.2±0.2	0.4±0.3	0.3±0.1	0.2±0.3	0.2±0.3
8	22.21	Cholesterol	2.9±1.8	2.0±1.3	3.4±1.6	2.2±1.2	3.3±2.9	1.6±1.3
9	22.29	3-MeC31	-	-	0.1±0.2	-	0.1±0.2	-
10	22.45	*n*-C32	*tr*	-	0.2±0.2	0.1±0.1	*tr*	*tr*
11	23.14	*n*-C33	0.8±0.5	0.9±0.5	1.0±0.6	1.1±0.6	1.3±0.6	1.2±0.6
12	23.37	11-/13-/15-MeC33	1.7±1.2	1.4±0.8	1.6±1.1	1.6±0.5	1.3±0.9	1.2±0.5
13	23.50	unidentified	-	0.8±0.8	0.2±0.4	0.7±0.5	*tr*	0.3±0.5
14	23.54	15,19-/13,x-/11,x-diMeC33	0.2±0.7	0.2±1.1	0.1±0.6	-	0.1±0.6	0.2±1.0
15	23.60	13,19-/11,21-/9,x-diMeC33	4.0±3.7	1.8±1.5	2.9±2.4	1.0±0.6	3.0±2.2	0.9±0.7
16	23.75	unidentified	0.3±0.7		0.1±0.3	-	*tr*	0.1±0.3
17	23.82	13,x,x-/11,x,x-/9,x,x-diMeC33	0.6±1.4	0.2±0.7	0.6±0.7	-	0.7±1.0	-
18	24.19	10-/11-/12-/13-/14-MeC34	0.2±0.4	0.1±0.3	0.5±0.6	-	0.1±0.2	-
19	24.44	unidentified	-	-	-	-	1.1±0.6	0.9±0.8
20	24.42	11,x-/12,x-/13,x-/14,x-diMeC34	*tr*	*tr*		-	-	-
21	25.09	11-/13-/15-/17-MeC35	5.7±1.9	5.6±1.9	6.1±2.2	6.1±1.3	4.6±1.6	5.0±1.5
22	25.27	unidentified	0.1±0.6	-	0.3±0.7	2.5±2.4	0.3±0.8	-
23	25.34	15,19-/13,17-/11,15-diMeC35	1.7±5.2	1.4±5.8	0.8±3.1	-	1.0±4.1	1.0±3.9
24	25.40	13,x-/11,23-/9,x-diMeC35	8.3±11.0	24.9±11.5	14.8±7.2	24.2±4.2	14.0±9.2	22.8±8.5

25	25.47	11,x-/9,x-/7,x-diMeC35	9.0±8.8	1.1±3.7	0.7±2.9	-	2.7±5.6	0.2±1.1
26	25.58	15,19,x-/13,17,x-triMeC35	0.8±2.6	0.7±2.9	0.4±1.5	-	0.4±1.6	0.4±1.5
27	25.61	13,17,x-/11,15,x-triMeC35	1.9±2.8	5.0±2.5	2.6±2.4	4.6±1.5	3.0±2.3	4.4±2.2
28	25.64	11,x,x-/9,x,x-diMeC35	2.0±2.5	0.2±0.6	1.0±1.7	-	0.6±1.3	-
29	25.89	3,x-diMeC35	0.2±0.6	-	0.3±0.4	-	0.3±0.5	-
30	26.47	12-/13-/14-/15-/16-MeC36	1.2±0.7	1.5±0.6	1.3±0.4	1.0±0.8	1.1±0.7	1.4±0.6
31	26.71	unidentified	*tr*	*tr*	*tr*	-	*tr*	-
32	27.38	11-/13-/15-/17-/19-MeC37	2.7±1.0	2.5±0.9	3.7±1.4	2.5±1.1	2.3±1.0	2.3±0.9
33	27.71	15,19-/15,21-/13,23-diMeC37	0.7±2.2	0.4±1.8	0.5±1.9	-	0.5±2.0	0.5±1.9
34	27.76	13,x- (17/23)/11,x-(25)/9,x-diMeC37	8.0±3.0	8.2±3.4	8.6±3.0	10.1±1.8	7.7±3.1	8.4±2.5
35	28.01	15,19,x-/13,17,x-triMeC37	0.4±1.1	0.2±0.9	0.3±1.2	-	0.3±1.3	0.3±1.0
36	28.05	13,17,x-/11,x,x-/9,x,x-triMeC37	0.9±1.4	1.7±0.9	1.2±1.3	1.9±0.9	1.1±1.1	1.6±1.0
37	28.13	11,15,x-/9,13,x-diMeC37	0.3±0.8	0.3±1.1	0.3±0.6	-	0.1±0.4	-
38	30.54	i-MeC39	*tr*	0.1±0.2	0.4±0.5	-	0.1±0.2	-
39	30.98	15,x-/13,x-diMeC39	0.1±0.3	0.2±0.5	0.2±0.6	0.2±0.4	0.1±0.5	0.2±1.2
40	31.05	11,x-(27)/9,x-diMeC39	0.8±1.2	0.1±0.4	1.4±1.5	-	1±1.4	0.1±0.3
		Number of compounds	16.7±1.8	16.9±1.6	21.2±2.3	18.3±2.1	18.1±2.2	16.9±1.9
		n-alkanes	44.6±7.6	39.0±10.1	43.4±7.4	40.0±6.6	47.9±7.1	45.6±8.4
		methyl-branched alkanes	11.5±3.8	11.3±3.7	13.9±4.1	11.3±2.0	9.5±3.5	9.8±2.7
		dimethyl-branched alkanes	32.8±5.5	38.4±8.5	30.1±5.1	35.5±5.1	30.0±5.7	34.4±7.2
		trimethyl-branched alkanes	6.5±4.0	8.0±3.6	6.0±2.8	6.5±2.4	6.0±2.9	6.7±2.6
		others	3.4±2.0	2.8±1.4	4.0±1.9	5.3±3.0	4.7±3.0	2.8±1.9

tr traces (<0.1%)

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Selbständigkeitserklärung

Hiermit erkläre ich, dass ich diese Dissertation selbständig und nur unter Verwendung der angegebenen Literatur und Hilfsmittel angefertigt habe. Die Dissertation ist in keinem früheren Promotionsverfahren angenommen oder als ungenügend bewertet worden. Die dem angestrebten Verfahren zu Grunde liegende Promotionsordnung erkenne ich an

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