Sexual Signals and Speciation

A Study of the Pied and Collared Flycatcher

BY

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Abstract

Speciation is the process in which reproductive barriers evolve between populations. In this thesis I examine how sexual signals contribute to the maintenance, reinforcement or breakdown of reproductive barriers.

Male pied flycatchers (Ficedula hypoleuca) and collared flycatchers (F. albigollis) differ in song and plumage traits. However, where the two species coexist, several pied flycatchers sing a song resembling the collared flycatcher (mixed song). Mixed song is not caused by introgression from the collared flycatcher but is due to heterospecific copying. Mixed song provokes aggressive behaviour in collared flycatcher males and leads to heterospecific pairing and maladaptive hybridization.

The species differences in song were found to be larger in an old than a young hybrid zone. This was due to a reduction in the frequency of mixed song in the pied flycatcher and a divergence in the song of the collared flycatcher. Apparently, mixed song causes maladaptive hybridization, which over time leads to reinforcement of reproductive barriers by a song divergence.

Previous studies have shown that a character displacement in male plumage traits reinforces species barriers. Hence both plumage and song divergence reduce the incidence of hybridization. The evolution of male plumage traits has been so rapid, or selection has been so strong that rapidly evolving molecular markers are unable to trace it.

Hybrid females mate with a male of the same species as their father. Previous studies have shown that females use male plumage traits controlled by genes linked to the sex chromosomes (the Z) in species recognition. An association between preference and a sex-linked trait through the paternal line may render reinforcement of reproductive barriers more likely.

In conclusion, sexual signals are affected by species interactions that cause breakdown or reinforcement of reproductive barriers.

Keywords: Speciation, Sexual signals, Hybridization, Reinforcement, Song, Female preference, Sex-linkage

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No problem can be solved from the same consciousness that created it

Albert Einstein
List of Papers


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Introduction

Theoretical and historical background

What are species?
The total number of species living on earth today is anyone’s guess, but current estimates range somewhere between 3 and 30 million. Considering that most of the species that have ever lived are extinct, the potential array of life forms seems almost unlimited. How did all this diversity evolve?

The answer to this question probably lays in the nature of species themselves. At first glance, species appear to be well-defined groups of individuals recognized by morphological differences. However, as any taxonomist is painfully aware of, if you look close enough, not two individuals are exactly similar. Earlier, taxonomists would consider individual variation as rather annoying deviations from a Platonic ideal – a “perfect” type. Later, however, it was realized that phenotypic variation was a real property of any species and thus that there was no such thing as a perfect type. This change, from a “typological” to a “populational” view of species, was a radical one. In fact, as Mayr (1959) put it: “no two ways of looking at nature cold be more different”. Individual variation within a population was now perceived as the very raw material for evolution and speciation.

On the origin of species
In the pre-Darwinian era species were defined somewhat loosely by their distinct differences in traits such as morphology, behaviour and colour. Thus, explaining the origin of species became equivalent of explaining the origin of such differences. Charles Darwin (1859) did this with his book “On the Origin of Species”. Individuals differ in traits and some of these are heritable. In the struggle for existence, individuals that exhibit favourable traits will leave more offspring, offspring that will exhibit these heritable
traits. In this process of natural selection organisms adapt to different environments. “How extremely stupid not to have thought of that!” responded T. H. Huxley after reading it. The idea was simple and as a matter of fact, Alfred Russel Wallace came up with the same idea independently. Together they published a paper in 1858, the year before the “Origin” (Darwin & Wallace 1858). Darwin was the first to realize that heritable individual variation was the origin of species. Even more revolutionary, he suggested that all living things (and extinct as well) had a common origin. Charles Darwin’s book is one of the most influential books in any field of science ever published. However, despite its title, it had surprisingly little to contribute to the understanding of how species originate.

A species concept based on trait differences has some severe limitations. Morphologically different individuals may be members of the same species. Worse, in species where males and females differ in morphology they may easily be mistaken as belonging to different species. The obvious solution to this problem was to observe whether or not two individuals would interbreed. Thus, it became clear that a more useful species concept would be “groups of actually or potentially interbreeding populations, which are reproductively isolated from other such groups” (Mayr 1942). This change of species concept, from one based on morphological differences to one based on reproductive isolation would change the view of speciation forever.

The geography of speciation
When a geographic barrier, such as a mountain range or a glacier, subdivides a population the populations will over time diverge due to natural selection, sexual selection and random events such as genetic drift. If the geographic barrier disappears, by the erosion of the mountain or the melting of the glacier, the daughter populations may come into secondary contact. If members of these populations interbreed successfully the populations will merge and the differences that accumulated in geographic isolation will blend. However, if members of the differentiated populations do not interbreed, or their hybrid offspring do not survive or reproduce, a biologist will consider the members of the populations to belong to distinct species. Thus, for species to maintain their identity, they have to be isolated from other species by “isolation mechanisms”, -barriers to gene exchange (Dobzhansky 1937; Mayr 1963). Darwin explained how natural selection creates adaptations, but not how reproductive isolation keeps species from blending together. The study of speciation thus became the study of how species barriers evolve. The questions were now: what are the “isolation mechanisms” and how do they evolve?
What keeps species apart?

The first species barrier to be recognized was the sterility of hybrids; the most famous example being the mule (the offspring of a female horse and a male donkey). However, sterility is only one of a number of species barriers. In birds, approximately one in ten species is known to hybridize but (according to some estimates) only one out of 60,000 individual birds is a hybrid (Mayr 1963; Grant & Grant 1992). Accordingly, barriers that prevent interbreeding from occurring in the first place are commonly what keep species apart. Species barriers are categorized as those that prevent two species from interbreeding (prezygotic barriers) and those that affect the hybrid offspring’s survival and fertility (postzygotic barriers) (Dobzhansky 1937). Thus, individuals of two species may never interbreed because they do not reproduce at the same time, because they use different habitats or resources and thus never meet, because they do not fancy each other as potential partners, because they differ in the structures of their reproductive organs or because their gametes do not match. If interbreeding does occur and the egg is fertilized, the hybrid offspring may not survive or reproduce.

How do “isolation mechanisms” evolve?

The most commonly held view is that speciation, the evolution of reproductive barriers, occurs by geographic isolation of populations. Reproductive barriers evolve, not because it is adaptive, but simply as a by-product of other processes (Muller 1940; Mayr 1963). However, if reproductive barriers are not complete when two differentiated populations come into secondary contact interbreeding can occur. If the hybrid offspring have reduced fitness, there is a cost of interbreeding. Thus, interbreeding would be “the grossest blunder [...] we can conceive an animal doing” because it could leave no or few reproducing descendants (Fisher 1930). As a consequence, natural selection should favour traits that reinforce species barriers to prevent hybridization from occurring (Dobzhansky 1937; Dobzhansky 1940). Intuitively, this sounds reasonable. However, this theory, named reinforcement in short (Blair 1955), implies that the evolution of reproductive barriers would by itself be an adaptive process evolving through natural selection. The theory of reinforcement of species barriers has been one of the most controversial theories in evolutionary biology (see e.g. Servedio & Noor 2003 for a recent review).

One of the major obstacles for reinforcement to occur is that even a very small leakage in the species barriers may prevent them from being reinforced and may even cause them to collapse, thereby causing the species to blend. In scientific terms, recombination will tend to break the necessary association between genes affecting pre- and postzygotic isolation
(Felsenstein 1981; Sanderson 1989). Worse, the higher fitness the hybrids have, the larger the leakage in the species barriers and the faster this breakdown will occur. Accordingly it is expected that the stronger linkage there is between genes controlling pre- and postzygotic isolation, the harder it is for recombination to hit the target and break the association (Felsenstein 1981; Servedio 2000). Just recently, empirical support for genetic factors promoting such linkage disequilibrium in species undergoing reinforcement has began to show up (Noor et al. 2001; Sætre et al. 2003) Moreover, reinforcement is further enhanced if these genes are linked on the macro sex chromosome (Servedio & Sætre 2003). This is because recombination is reduced with sex-linked inheritance since it only occur in the homogametic sex (XX or ZZ), and because selection can be more effective on sex-linked genes since recessive alleles with a bearing on fitness are not masked by dominance in the heterogametic sex (Servedio & Sætre 2003). The theory of speciation by reinforcement was founded more than 60 years ago (Dobzhansky 1937; Dobzhansky 1940) but its popularity has “resembled stock market fluctuations” (Noor 1999). The theory has inspired the generation of a large amount of theoretical models; most of them showing that reinforcement could work under some more or less restricted conditions (Servedio & Noor 2003). It went so far that a biologist wrote, “...it has become clear that the issue cannot be settled by theoretical arguments but by evidence from natural populations...” (Howard 1993). Today, convincing examples of reinforcement has been published in studies of frogs, snails, fruit flies and birds (Littlejohn 1965; Johannesson et al. 1995; Noor 1995; Sætre et al. 1997b). Thus, the question is no longer whether or not reinforcement can happen, but how important it is in speciation and under which conditions it occurs (Servedio & Noor 2003).

Sexual signals and speciation
“The sight of a feather in the peacock’s tail, whenever I gaze at it, makes me sick” Darwin wrote just a year after the publication of the “Origin”. What Darwin feared was that when he looked into the beauty of the peacocks tail, he saw the Nemesis of his own theory. To Darwin, such elaborated traits seemed opposite from what he would expect from evolution by natural selection. Such traits must be expensive to develop, sometimes dangerous to carry, and obviously useless in the struggle for survival. Later, Darwin realized that what he saw had nothing to do with survival but all to do with sex (Darwin 1871). Much later it was realized that such extravagant traits might even have a lot to do with the origin of species.

Sexual selection “arises from differences in mating success” (Arnold 1994). Males produce an incredible amount of tiny gametes, while females only produce a few large gametes. Consequently, males have the potential to
father many more offspring than females. Therefore, males would tend to compete to get access to females whereas females would tend to be coy in their choice of mates and to preferentially choose those with traits that indicate that they have resources, are good at paternal care or have “good genes” (Darwin 1871; Fisher 1930; Zahavi 1975; Hamilton & Zuk 1982). As a consequence, some males with some desirable trait may sire a larger proportion of offspring. The outcome of sexual selection can be sexually dimorphic traits such as weapons, e.g. horns and tusks, and elaborated show-off traits, such as the peacock’s tail. The latter traits function as sexual signals used by one sex to attract members of the other sex and such communication systems often function as isolation barriers between species (Paterson 1985). Thus, species recognition may simply be the consequence of finding the best mate (Littlejohn 1998). If two isolated populations diverge in the cues and preferences used in mate recognition, speciation has occurred when males and females of two populations do not recognize each other as potential mates. Thus, sexual selection could cause two populations to diverge in traits that would later function as species recognition cues (Lande 1980; Lande 1981). Closely related species often differ most strikingly in sexually selected traits and the most species rich taxa are often more strongly sexually selected than others (see references in Price 1998). These observations indicate that sexual selection may play an important role in the evolution of reproductive isolation.

It should be noted, however, that sexual selection is not the only way in which sexual signals can diverge. Natural selection and random drift may cause sexual signals to diverge in geographic isolation. For communication to work you have to make sure that you are seen, heard or smelled. A signal that functions well in one environment might be quite useless in another. A green plumage colour may be striking against the blue sky above but cryptic against the green grass below. Thus, natural selection for efficient signal transmission can cause two populations that live in different habitat to diverge in sexual signals (Endler 1992; Schluter & Price 1993; Sætre 2000). Examples of natural selection shaping signals include birds adjusting the frequency of their song to different acoustic environments (Morton 1975; Wiley 1991; Slabbekoorn & Smith 2002).

In birds, song is often used in species recognition (for references see Ptacek 2000). Since learning often plays an important role in song development (ten Cate & Vos 1999), small errors in transmission can over time give rise to larger population differences. It has been suggested that such random drift can give rise to song dialects that can function as reproductive barriers between populations (Marler & Tamura 1962; Baker & Mewaldt 1978; Grant & Grant 1996). However, the role of song in speciation remains controversial (Raikow 1986; Baptista & Trail 1992; Salomon & Hemim
Natural selection, sexual selection and random events can cause two populations to diverge in sexual signals. However, although populations may differ in male sexual traits, females may not necessarily have different preferences (Endler & Houde 1995). Thus, for reproductive barriers to evolve there has to be a corresponding divergence in female preference (Price 1998). When female preferences diverge, sexual preferences may become preferences used in species recognition (Mayr 1992). Therefore, divergence in female mate preferences is clearly a key process in speciation. Yet the nature and control of such preferences are rarely studied, probably because it is difficult to do so. For instance, it has only rarely been investigated to what extent a female preference for a trait is genetically controlled or determined by learning processes such as sexual imprinting. Studying female preferences therefore remains a large challenge for students of sexual selection and speciation.

The challenge

Now, almost 150 years after Darwin published the “Origin”, a reviewer stated that “speciation is one of the least understood major features of evolution” (Schluter 2001). This does not mean that there has not been progress in the field of evolutionary biology; rather, research has shown that the complexity and diversity of how speciation occurs is far greater than anyone could guess. The problem is to recognize which processes that cause speciation (Schluter 2001). The role of sexual selection in speciation is currently an area of active research. However, one obstacle to further progress is to understand why females choose as they do. Accordingly, today the sight of a feather in the peacock’s tail would probably not make an evolutionary biologist sick, but it might still induce a slight feeling of discomfort. Sexual signals are probably the single most important species barrier in animals. In fact, in some cases, such as in various species of birds, they may virtually be the only barrier against gene flow (Grant & Grant 1997). Sexual signals are affected by natural selection, sexual selection and random drift, but to show that these forces are the causes of reproductive barriers remains a challenge (Schluter 2001).

In this thesis I address some of the major issue in evolutionary biology such as: What are the factors that contribute to the maintenance, reinforcement or breakdown of reproductive isolation. What is the role of sexual signals and mate choice in the evolution of reproductive barriers? How does genetic architecture and learning mechanisms affect the likelihood of speciation by reinforcement? How fast does reproductive barriers evolve? The best
approach to address these questions empirically is probably to study the “natural laboratories” of evolutionary processes related to speciation (Arnold 1997), namely natural hybrid zones.
Study species

The pied flycatchers (*Ficedula hypoleuca*) and collared flycatchers (*F. albicollis*) are two closely related passerine bird species (or semispecies) belonging to the family Muscicapidae that are known to hybridize. Pied flycatcher males are extremely variable in plumage traits, ranging from conspicuous black-and-white to female-like brown as illustrated on the front cover (Drost 1936). Collared flycatcher males are black and white but easily distinguishable from the pied flycatcher males by a distinct white neck collar and larger white patches on the forehead and the wings. In contrast to the well-defined species differences in male plumage traits, females of the two species are very similar. Collared flycatcher females are somewhat greyer in overall plumage colour compared to the warmer brownish colour of pied flycatcher females and the former also have larger white patches on the base of their primaries. The song of pied and collared flycatcher males is complex (Lundberg & Alatalo 1992), but easily distinguishable in the field, mainly through the higher frequency and the lower tempo of the collared flycatcher (Gelter 1987). However, in populations where pied and collared flycatchers coexist (sympatry) some pied flycatchers have a song that resembles that of the collared flycatcher (see below). The two species also differ in alarm calls, pied flycatchers uttering a short “pit” and collared flycatchers a prolonged, sad-sounding “eiip”.

The pied flycatcher is found breeding over larger parts of Europe, whereas the breeding distribution of the collared flycatcher is limited to Italy, Central and Eastern Europe, and the Baltic Islands of Öland and Gotland off the Swedish East coast (Fig 1.). Hybridization occurs where the two species coexist, but is limited to a rather narrow zone corresponding to topography and habitat (Alerstam et al. 1978; Sætre et al. 1999). The hybrid zone in Central Europe is assumed to be of secondary contact after the Pleistocene glaciations (von Haartman 1949; Sætre et al. 2001a) while the Swedish hybrid zone is apparently of more recent origin, possibly only about 150 years old (Alatalo et al. 1990).

Hybrids are recognized by their intermediate plumage traits, song and alarm calls. Most striking is the incomplete collar of male hybrids. Female hybrids are almost impossible to recognize by plumage traits alone. The song of hybrid males is intermediate but appears more similar to collared than to pied flycatcher song (Gelter 1987). The alarm calls of hybrids are typically random alternations between both species calls (Gelter 1987).
Sexual signals and hybridization

In areas where the breeding distribution of pied and collared flycatchers overlap, hybridization occurs occasionally (2-7% of the breeding pairs being mixed-species pairs in different sympatric populations, Alatalo et al. 1982; Sætre et al. 1999; Veen et al. 2001). Although females of the two species are slightly different to the human eye, they are similar to the extent that males of both species appears to be unable to distinguish between them (Sætre et al. 1997a). Accordingly, assortative mating is based on female mate choice (Alatalo et al. 1982; Sætre et al. 1997b). What is then the cause of hybridization? Three explanations have been suggested for why females might mate with a heterospecific. Females may lack alternatives, be confused about species identity or benefit from mating heterospecifically.
In the study populations, collared flycatcher outnumbers the pied flycatcher. Thus, it is not surprising that pied flycatcher females may sometimes be forced to mate with collared flycatcher males when they find it hard to find conspecifics. However, the collared flycatcher females are just as likely to be involved in mixed mating as pied flycatcher females (Alatalo et al. 1990; Sætre et al. 1999). Thus, a lack of conspecifics does not sufficiently explain the occurrence of mixed mating involving collared flycatcher females.

Pied flycatcher males that are involved in mixed mating with collared flycatchers are more similar to collared flycatchers in plumage traits (more brightly coloured) (Alatalo et al. 1994) and possibly also in song (mixed song) (Alatalo et al. 1990; Eriksson 1991). Thus, resemblance in plumage traits and song seems to increase the likelihood of mixed pairing involving collared flycatcher females. Both the lack of conspecifics and confusion in mate recognition may explain the occurrence of mixed pairing between the two flycatcher species.

Breeding success declines dramatically during the breeding season in both species (Veen et al. 2001). However, pied flycatcher males appears to be better than collared flycatchers at providing resources to nestlings later in the breeding season (Wiggins et al. 1994; Veen et al. 2001). Thus late arriving collared flycatcher females may increase the direct benefits to their offspring by mating with a pied flycatcher male. Apparently, collared flycatcher females also reduce the cost of mixed mating by seeking extra-pair-copulations (EPCs) with conspecific males. In fact, more than half the offspring in these mixed pairs are sired by collared flycatcher males (Veen et al. 2001). Hence, late arriving collared flycatcher females may actually benefit from pairing with pied flycatcher males (Veen et al. 2001).

### Sexual signals and speciation

Hybrids have lower fitness than pure pied and collared flycatchers mainly due to reduced fertility. Females are almost entirely sterile whereas males are at least partly fertile (Alatalo et al. 1982; Gelter et al. 1992; Sætre et al. 1997b; Sætre et al. 1999; Sætre et al. 2003), a pattern consistent with Haldane’s rule (Haldane 1922).

This reduction in hybrid fitness represents a significant selection potential for reinforcement of prezygotic isolation to occur (Dobzhansky 1937; Dobzhansky 1940). Indeed, convincing evidence for reinforcement has been obtained in the case of these flycatchers (Sætre et al. 1997; 2003). The two species have more similar plumage characteristics in allopatry than in sympatry. Experimental evidence shows that in sympatry this divergence in male plumage traits is driven by divergence in female mate preferences.
Accordingly, the resulting character displacement reduces the frequency of maladaptive hybridization (Sætre et al. 1997b). Pied flycatcher males that are most different in plumage traits compared to collared flycatcher males run a smaller risk of getting involved in hybridization, an observation that support this scenario (Alatalo et al. 1994).

Interspecific competition is an alternative explanation that has been proposed for the cause of the plumage divergence in sympathy. Collared flycatcher males are socially dominant over pied flycatcher males in competition for territories and nest holes (Löhrl 1955; Alatalo et al. 1982). Experiments have shown that resident collared flycatcher males show less aggression towards brown than black dummies of pied flycatcher males, presented at their nest-box (Kral et al. 1988; Røskaft & Järvi 1992). Because brown males resemble females in plumage traits, it has been suggested that pied flycatcher males suffer less aggression from collared flycatcher males by an interspecific female mimicry strategy (Røskaft & Järvi 1992; Sætre et al. 1993). Indeed, resident collared flycatcher males responded to female-like brown pied flycatcher males with a similar mate attraction behaviour as towards prospecting females (Sætre et al. 1993). Accordingly, male collared flycatchers seem unable to distinguish between intruding male pied flycatchers and prospecting females, at least when the former do not reveal their sex by song. However, given that the collared flycatcher is dominant in interspecific competition with the pied flycatcher, interspecific aggression cannot explain why the collared flycatcher males have a more divergent plumage in the areas where the two species coexist (Sætre et al. 2003).

To conclude this section, there is evidence suggesting that maladaptive hybridization has played a significant role in causing the observed plumage divergence in the flycatchers. In addition, interspecific competition may have contributed to this divergence through synergistic effects of female mimicry in the pied flycatcher. However, how this apparent confusion in sex- and species recognition affects interspecific competition and to what extent it is adaptive remains to be tested.

Male plumage traits are under sexual selection in allopatry by female mate choice and male-male competition (Sætre et al. 1994; Gustafsson et al. 1995; Pärt & Qvarnström 1997; Qvarnström 1997; Qvarnström & Pärt 1997; Sheldon & Ellegren 1999; Qvarnström et al. 2000). Incipient speciation occurred during the Pleistocene glaciation periods (Sætre et al. 2001b) and it is likely that sexual signals began to diverge in geographic isolation. When the two species came into secondary contact sexually selected traits reinforced the species barriers (Sætre et al. 1997b; Sætre et al. 2003). Reinforcement in the flycatchers has been described as one of the most convincing evidence for reinforcement yet published (Butlin 1999).
Mixed song

Where the two species coexists, a large proportion of pied flycatchers are known to sing a song resembling that of the collared flycatcher a phenomenon known as mixed song (Tischler 1942; Löhrl 1955; Gelter 1987; Eriksson 1991). On average, mixed song is intermediate between the typical song of the pied and collared flycatcher in both characters and content (Gelter 1987; Eriksson 1991). However, it should be noted that there is large individual variation. Some males sing a song that is almost indistinguishable from collared flycatcher song while others have only a weak resemblance (Gelter 1987; Eriksson 1991). Thus, mixed song is not a discrete category but the term will be used here for its heuristic value.

Males attract females by their song and some evidence suggest that song may also play a role in male-male competition over territories (Eriksson 1986; Eriksson 1991). Females prefer to mate with males that exhibit a large song repertoire (Eriksson 1991; Lampe & Sætre 1995) and song has been suggested to signal the health status and condition of the male (Lampe & Espmark 1994; Garamszegi et al. 2003; Lampe & Espmark 2003).

One study suggests that song may be used in species recognition, but the support for this conclusion was rather indirect (Lundberg & Alatalo 1992). It has been suggested that pied flycatchers may benefit in competition over territories with the collared flycatcher by singing a mixed song (Eriksson 1991). However, they may also suffer an increased risk of hybridization (Alatalo et al. 1990; Eriksson 1991).

As reviewed above, several lines of evidence suggest that sexual signals, both plumage traits and song, in the pied and collared flycatchers, play a role in species recognition and have been affected by interspecific interactions. However, the study of the role of song in hybridization and speciation has remained a challenge. Furthermore, the association between species-specific male traits and female mate choice is also largely unexplored.
Research aims

The focus of this thesis is on the sexual signals that contribute to the maintenance, reinforcement or breakdown of reproductive isolation in hybrid zones. The sexual signals that I have studied are male plumage and song characteristics and the behavioural responses to these signals. More specifically I ask:

- What are the causes and consequences of mixed song in pied flycatchers?
- How fast can reproductive barriers, such as species-specific male plumage traits, evolve?
- What determines female preference for species-specific traits and how does it affect speciation?
Results and discussion

Mixed song is not caused by introgression (paper I)

Gene flow between two species (introgression) will over time cause them to blend. Accordingly, introgression may also cause genetically determined sexual signals to converge. Thus, it could be expected that introgression from collared flycatchers could be the cause of mixed song in the pied flycatcher. A high-resolution DNA analysis (SNPs) was used to study the effect of introgression on the song of pied and collared flycatchers.

Pied flycatchers that were classified as singing a mixed song did not possess any alleles introgressed from the collared flycatcher. Furthermore, collared flycatchers that possessed pied flycatcher alleles did not differ in song characteristics from those that had a “pure” collared flycatcher genotype. Previous studies have shown that introgression occurs almost exclusively from pied to collared flycatchers and not vice versa as could be expected if introgression was the cause of mixed song (Sætre et al. 2003). Thus, there is evidence that introgression does not affect the song of the pied and collared flycatchers.

Figure 2 A figure illustrating the culture and gene flow between the pied and the collared flycatcher. Heterospecific song learning (mixed song) occurs in the pied flycatcher, but gene flow occurs predominately from the pied to the collared flycatcher.
In song birds (Oscines) the song usually develops through learning, often under genetic constraints by an innate auditory song template (Kroodsma 1982; Baptista 1996). Since introgression does not appear to affect the song of either species, this implies that heterospecific learning is the dominant proximate factor explaining the occurrence of mixed song in the pied flycatcher (Fig 2). The pied flycatcher may have rudimentary collared flycatcher structures in their song-learning predisposition (Eriksson 1991). Thus, social interactions with the collared flycatcher are presumably the cause of mixed singing (Eriksson 1991).

Mixed song cause interspecific aggression (paper II)

Song often has a dual function in communication since it is used both in mate attraction and male-male competition (Catchpole & Slater 1995). Interspecific competition and aggression may cause species-specific signals to change. Playback experiments were conducted to study the aggressive response of collared flycatcher males to mixed song compared to pure pied and collared flycatcher song.

![Figure 3 Aggressive responses of male collared flycatchers to playback of collared or pied (mixed or pure) song. Collared flycatcher males responded aggressively (by attacking the dummy) more often to collared than to pure pied flycatcher song and more often to mixed than to pure pied flycatcher song.](image)

**Figure 3** Aggressive responses of male collared flycatchers to playback of collared or pied (mixed or pure) song. Collared flycatcher males responded aggressively (by attacking the dummy) more often to collared than to pure pied flycatcher song and more often to mixed than to pure pied flycatcher song.
Collared flycatcher males responded aggressively more often to mixed than to pure pied flycatcher song (Fig. 3.). In fact, the response was as strong as if the playback song was from a collared flycatcher. Thus, collared flycatcher males seem to respond to mixed song as if it was from a conspecific, a result consistent with earlier findings (Eriksson 1991). Song convergence may facilitate interspecific territoriality enabling two competing species to coexist (Cody 1974). Heterospecific song copying has been suggested to lead to better competitive abilities of the socially subdominant species (Sorjonen 1986). Since the pied flycatcher is socially subdominant to the collared flycatcher in competition for territories (Löhrl 1955; Alatalo et al. 1982), mixed singing might be expected to compensate for this disadvantage (Eriksson 1991). Also, if pied flycatcher males are rare, collared flycatchers might not recognize their song. Thus, mixed song may facilitate interspecific communication. Although mixed song induced a stronger response in collared flycatcher males, it does not follow from these playback experiments that it is adaptive. The counter-argument, that mixed song could be a disadvantage in interspecific competition because collared flycatcher males respond more aggressively, could just as well be valid. Clearly, further experiments are needed to unravel the benefits or costs of mixed song in interspecific competition.

It has been suggested that song-matching, i.e. the matching of the song of neighbours, may have a function in male-male competition over territories (Krebs et al. 1981; Catchpole & Slater 1995). Thus, in areas where pied and collared flycatchers coexist, pied flycatchers may conceive collared flycatchers as competitors and consequently copy their song. Accordingly, the origin of mixed song may also be song matching, but there is currently no evidence to support this hypothesis.

Mixed song leads to hybridization (paper II & III)

In birds, song is often involved in species recognition (reviewed by Ptacek 2000). However, since normal song development often involves learning from conspecifics it may be more plastic in its expression than other sexual signals. Accordingly, species interactions may cause males to learn a heterospecific song leading to confusion in species recognition. I tested whether mixed song in the pied flycatcher increases the probability of hybridization.
Figure 4 Pied flycatcher males singing mixed song more often paired with a heterospecific female than males singing pure song.

Figure 5 First and second principal component of pied and collared flycatcher song extracted from 9 different song variables. Also indicated are those pied flycatchers scored in the field as having mixed song and those that mated heterospecifically.
Female collared flycatchers mated with pied flycatchers males only if they sang a mixed song (Fig. 4.). Furthermore, the more similar the mixed song was to the collared flycatcher song, the more likely the male was to be mated with a collared flycatcher female (Fig. 5.). Accordingly, the results indicate that song is a trait used in species recognition and that pied flycatcher males singing a mixed song have a higher probability of pairing with collared flycatcher females. Previous studies have shown that mixed mating leads to hybridization (Veen et al. 2001). Pied flycatcher males that are involved in mixed mating with collared flycatchers have also been found to be more similar to collared flycatchers in plumage traits (more brightly coloured) (Alatalo et al. 1994), a pattern that is also experimentally confirmed (Sætre et al. 1997b). Thus, resemblance in both plumage traits and song seems to increase the likelihood of pairing with collared flycatcher females and consequently hybridization.

One objection that could be made to this interpretation of the results is that mixed song may not lead to mixed mating, but simply develop where mixed mating is most likely to occur. Thus, to establish a causal link between mixed song and heterospecific pairing, I conducted a playback experiment using aviaries. In this experiment the response of collared flycatcher females to mixed pied flycatcher song was compared to the response to pure pied and collared flycatcher song. Unfortunately, females did not respond to the playback song. One explanation for this outcome could be that song is a long distance signal of species identity. Song may function as a first filter in species recognition. However, females may use other traits, such as male plumage trait, upon making her final choice. Furthermore, song may on its own be a too weak stimulus to induce a female response with the experimental setup used. Previously, hormone implanted flycatcher females have been used to study song discrimination with some success (Eriksson 1991; Lundberg & Alatalo 1992). However, it is uncertain to what degree this experimental setup reflects a natural situation. Another potential problem in these studies (i.e. Paper III, Eriksson 1991) is that female nest-box visits were used to estimate female mate preferences, whereas actual nest building may be a more reliable measurement (Sætre et al. 1994; Lampe & Sætre 1995; Sætre et al. 1997a). The best method in addressing questions related to the role of song in species recognition might actually be field experiments (e.g. Eriksson 1986).
Geographic song variation, convergence, divergence and reinforcement (paper I)

Song is used in species recognition. It has therefore been suggested that song divergence could cause reproductive isolation between populations (Marler & Tamura 1962; Baker & Mewaldt 1978). However, it is unclear whether selection against maladaptive hybridization can cause species barriers to be reinforced by song divergence (see Price 1998 for a discussion). One important test is to study hybridizing species to see if species differences in song converge or diverge in sympatry. To address this question, songs of pied and collared flycatchers from allopatric and sympatric populations were compared. Two hybrid zones of different ages were compared to study how species interactions may affect the song over time.

The results show that pied and collared flycatchers differ in song in allopatry and sympatry. The species difference in song is larger in the old than in the young hybrid zone (Fig. 6). This is probably due to a reduction in the frequency of mixed song in the pied flycatcher and a divergence in the song of the collared flycatcher. In both hybrid zones, collared flycatchers seem to have diverged in the song characters that the pied flycatchers copy in their mixed song.

Figure 6 Species differences in the song of pied and collared flycatcher in an a) old and a b) young hybrid zone. The species differences are larger in the old hybrid zone.
Figure 7 A scenario of the species differences in song of the pied and collared flycatchers. Due to mixed song the species differences decrease when the two species come into secondary contact (early sympatry). Mixed song leads to maladaptive hybridization, thus over time natural selection cause a reinforcement of species barriers by a song divergence (late sympatry).

A possible scenario could be that, when the two species came into secondary contact, heterospecific copying in the pied flycatcher cause the species differences in the song to decrease (Fig. 7). Accordingly, mixed song may increase the frequency of hybridization. Natural selection against maladaptive hybridization may then favour traits that increase assortative mating, for instance by a divergence in an innate auditory template. Thus over time the species differences increase and the frequency of hybridization decrease. The two hybrid zones studied here may represent different stages in the speciation process. It could be speculated that if heterospecific copying increases the frequency of hybridization, it may increase the selective pressure for reinforcement of prezygotic barriers. Thus, culturally determined traits used in species recognition may, under certain circumstances, function as a catalyst in speciation. Whether a culturally inherited trait promotes or opposes speciation in sympatry may depend on its plasticity. The degree of plasticity may be genetically determined and thus under selection by reinforcement (Irwin & Price 1999).
Reinforcement can occur rapidly and/or strongly (paper IV)

Phylogenetic analyses suggest that the pied and collared flycatcher split from a common ancestor during the Pleistocene glaciation (Sætre et al. 2001a) about 2 million years ago (Price & Bouvier 2002). However, much more recently, probably after the last glaciation period about 10,000 years ago, the pied and collared flycatcher came into secondary contact. Apparently, reproductive barriers by a reproductive character displacement in male plumage traits have evolved to prevent maladaptive hybridization (Sætre et al. 1997b; Sætre et al. 2003). What is the evolutionary rate of the plumage colour evolution compared to rapidly evolving molecular markers?

An allopatric Norwegian and a sympatric Czech pied flycatcher population did not differ in allele frequencies at molecular markers (microsatellites and mtDNA). Yet, males in the two populations are very different in plumage characteristics, Czech males being significantly browner than Norwegian ones. Thus, there is a discrepancy between plumage and genetic differentiation. Recent isolation or gene flow may be the cause of the genetic similarity. Apparently, the evolution of male plumage traits has been so rapid, or selection has been so strong that rapidly evolving neutral molecular markers are unable to trace it.

Is gene flow from sympatry the cause of the large plumage variation in allopatry? In an allopatric Spanish population, genetically isolated from sympatric populations, brown males also occur. Thus gene flow from sympatric populations is not necessary to maintain the large plumage variation in allopatric pied flycatchers. Other mechanisms, such as predation, may maintain the variation (Slagsvold et al. 1995). Consequently, the data suggest that the brown plumage of sympatric pied flycatchers may be originally derived from the large plumage variation found in allopatric pied flycatchers.

Paternally determined species recognition (paper V)

The theory of reinforcement predicts that when maladaptive hybridization occurs, natural selection should favour traits that increase assortative mating. However, recombination may cause the genes that are involved in assortative mating (prezygotic barriers) to become randomly combined with those that reduce the fitness of the hybrids (postzygotic barriers) (Felsenstein 1981; Sanderson 1989). Thus, for reinforcement to work, genes controlling pre- and postzygotic barriers have to be linked. Hence, when sexual signals are used in mate recognition, mating signal, preference and hybrid sterility have to be associated for reinforcement to operate. Only a few studies have
demonstrated such linkage (Noor et al. 2001) and in general surprisingly little is known about the control of female mate preferences. I examined the association between the male and female component of mate recognition by studying mate choice of female hybrids. To infer the paternal and maternal species identity of the hybrid, sex- and species specific genetic markers were used.

Hybrid females mate with a male of the same species as their father (Fig. 8). Accordingly, species recognition is paternally determined either genetically or through sexual imprinting. According to a recent study, genes determining male plumage traits used in species recognition and genes causing hybrid sterility are both linked to the Z-chromosomes (Sætre et al. 2003). Thus, the female component of species recognition is effectively linked to the Z-chromosome. Consequently, genes affecting pre- and postzygotic barriers are linked on the sex chromosomes. In a recent theoretical paper, Servedio & Sætre (2003) demonstrate that sex-linkage of genes controlling pre- and postzygotic isolation greatly enhance the reinforcement process.

Figure 8 Hybrid females mate predominately with males of the paternal species.
Conclusion

In birds sexual signals are probably the most important species barrier. Understanding speciation is thus closely associated with the understanding of how sexual signals contribute to the maintenance, reinforcement or breakdown of reproductive isolation. In the pied and collared flycatchers, male song and plumage traits are involved in species recognition. Song is affected by learning and species interactions leads to mixed song in the pied flycatcher. Song is used in female mate choice and may also be involved in male-male competition over territories. Hence, mixed song may facilitate aggressive signalling between heterospecific males but may also lead to heterospecific mating and hybridization. Both the lack of conspecifics and confusion in mate recognition may explain the occurrence of mixed pairing between the two flycatcher species. Patterns of geographic song variation suggest that divergence in song may reinforce species barriers. Molecular analyses suggest that such reproductive barriers can evolve rapidly. Species recognition in females is paternally determined either genetically or through sexual imprinting and sex linkage of both pre- and postzygotic barriers may promote reinforcement.
Future prospects

Sexual signals have been thoroughly studied in the pied and collared flycatcher. Sexual selection, hybridization, and interspecific competition have all been suggested to play a role in shaping sexual signals. One of the major challenges is to separate the effects of these forces in the evolution of sexual signals. In the case of male song, more experiments are urgently needed to test the importance of song in species recognition and interspecific competition. One difficult task of future research is to establish the generality of these results. In some aspects the pied and collared flycatcher may represent a special case in the evolution of sexual signals. For instance, no other European bird species exhibit so much plumage colour variation as the pied flycatcher. Mixed song is rare and usually only occurs at a very low frequency in various species of birds, but in the pied flycatcher it is extremely frequent. Furthermore, few other avian hybrid zones with such high frequency of hybridization exist. Finally, strong intrinsic postzygotic barriers to gene flow as found in the flycatchers appear to be rare among closely related bird taxa. These attributes of the flycatcher model system are the features that make them unique in addressing evolutionary questions and so exciting to study.

The findings in this thesis raise several questions that bother a curious mind after the odyssey of a PhD. When do pied flycatchers learn their song? Is mixed song beneficial in interspecific competition? Why is there no mixed song in the collared flycatcher? Is there a lack of ability or opportunity? Why is mixed song such a rare phenomenon in nature? If song is an unreliable cue of species identity, why don’t females use more reliable traits such as plumage traits? What is the role of competition versus hybridization in shaping sexual signals? If plumage divergence reinforces species barriers, why is there a similar divergence in song? Can females discriminate between songs of males from allopatric and sympatric populations? How does reinforcement affect the plasticity of traits? Does learning promote or oppose speciation in general and by reinforcement in special? Is female preference genetically determined or does it develop though imprinting? What is the relationship between sexual signals, sexual selection, sex chromosomes and speciation? And what is the meaning of life?

*The important thing is not to stop questioning, Albert Einstein*
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A doctoral dissertation from the Faculty of Science and Technology, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology. (Prior to October, 1993, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science”.)