# The contributions of seasonality, song and hormonal regulation to the evolution of songbirds

**Dissertation submitted for the degree of**
**Doctor of Natural Sciences**

Presented by
**Kim Geraldine Mortega**

at the

Universität
Konstanz

Faculty of Sciences
Department of Biology

June 22nd 2015
PD Dr. Barbara Helm
Prof. Dr. Michaela Hau
Prof. Dr. Karl-Otto Rothhaupt

*For my parents*

*Und jedem Anfang wohnt ein Zauber inne,*
*Der uns beschützt und der uns hilft, zu leben.*
*Hermann Hesse*

The University Of Sheffield.
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MAX-PLANCK-GESELLSCHAFT
International Max Planck Research School for Organismal Biology
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University of Glasgow

PhD defense for the degree of Doctor of Natural Sciences

# THE CONTRIBUTIONS OF SEASONALITY, SONG AND HORMONAL REGULATION TO THE EVOLUTION OF SONGBIRDS

Presented by
**Kim Geraldine Mortega**

**Monday June 22nd 2015**
**10.30 a.m.**

at the

University of Konstanz

Department of Biology
Limnology
U206
Mainaustrasse 252
78464 Konstanz / Egg

Invited Guestspeakers

at the
Max-Planck-Institut für Ornithologie
Radolfzell
2.30 p.m.

**Jan Christian Habel**

Technische Universität München

"Small scale short term and large-scale long term population differentiation in East African bird species"

and

**Rosemary & Peter Grant**

PRINCETON UNIVERSITY

“Darwin’s Finches:
From field ecology to molecular genetics”

Examination board
PD Dr. Barbara Helm
Prof. Dr. Michaela Hau
Prof. Dr. Karl-Otto Rothhaupt

DFG

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**Re: Assessment of the Ph.D. thesis on "The contributions of seasonality, song and hormonal regulation to the evolution of songbirds" by Kim Geraldine Mortega**

21. May 2015

Kim Mortega has submitted an excellent Ph.D. thesis, for which she has collected unique empirical data to examine the ecological and behavioral factors underlying population and species differentiation in two wild songbird species. Kim's thesis is comprised of seven chapters, five of which have already been published in international, peer-reviewed journals, with Kim being first author on two and holding a joined first-authorship on a third one. The two remaining chapters are in a manuscript form but are entirely publishable as well.

In her thesis, Kim combines multiple cutting-edge techniques and uses both experimental and observational approaches to characterize the extent of behavioral, genetic, and endocrine divergences among populations across a gradient of distances, from fairly close proximity up to a continent apart. She collected a wealth of unique data, applied superb statistical tools and embedded her findings into the context of current concepts. She collaborated with a number of both junior and senior colleagues in- and outside of her group and integrated a wide variety of approaches into her work.

In Chapter 1, Kim examined the geographical variation in sexually selected traits, i.e. male morphology and song in a migratory songbird, the stonechat *(Saxicola torquata)*. For this, Kim first quantified differences in male song among stonechat populations from central Europe and Africa. Second, she tested whether stonechats from a German population would be able to discriminate between song playbacks and decoys from their own versus two other central European populations and an African population.
Different populations indeed differed in song traits. Furthermore, individuals from a German population were able to discriminate between songs and decoys from other stonechat populations, responding less strongly to these stimuli with increasing geographical distance. Thus, stonechats have the ability to distinguish song and morphological traits from a local compared to distant populations. This ability could be one of the key behavioral processes that over time may lead to increasing differentiation among populations, potentially even resulting in speciation.

In Chapter 2, Kim and her co-authors studied the endocrine mechanisms underlying the expression of song and territorial aggression in black redstarts *(Phoenicurus ochruros)* during both the breeding and the non-breeding seasons. While song and aggressive behavior that male birds display during the breeding season is often considered to be regulated by testosterone, black redstarts show these behaviors also during the non-breeding season, when the testes, the main testosterone-producing organs, are regressed.
Kim's data show that during the non-breeding season male black redstarts sing at a rate equal to that during the breeding season, but that their song contains less repetitive elements during the non-breeding season. Territorial behavior is equally strong, and the volume of a brain area involved in song is equally sized in both breeding and non-breeding seasons. However, there were seasonal differences in the expression of steroidogenic enzymes in certain brain areas suggesting seasonally divergent local production of steroid hormones. Taken together Kim's data suggest that general territorial behaviorincluding song is independent of testosterone in this species, but that increased testosterone concentrations may facilitate context-dependent changes in song traits during the breeding season in male black redstarts.

Following up on this work, in Chapter 3 Kim and her co-authors experimentally tested whether in black redstarts territorial aggression and song during the breeding season are indeed regulated by testosterone by treating free-living males with pharmacological blockers of testosterone action. By recording behavioral responses at two time points after the treatment, Kim demonstrated that although the overall territorial response was not reduced in treated males, they put a greater effort into displays directed towards the intruder whereas control-treated males showed a vocal response that was likely more directed towards their mates. These data confirm that even during the breeding season, testosterone appears not necessary for the expression of territorial behavior in black redstart males, but that it functions instead to facilitate changes in song related to reproductive purposes.

With Chapter 4, Kim concluded the topic of the endocrine control mechanisms of song and territorial aggression in black redstarts. Here she and her co-authors compared the effectiveness of testosterone blockers when they are administered during the breeding versus the non-breeding season. When challenged with a simulated territorial intrusion via the presentation of a decoy and song playback of aconspecific male, blocker-treated males modulated other aspects of their song type compared to control-treated males. Interestingly, in fall, at a time when testosterone levels are naturally low, males from bothtreatment groups displayed the song patterns shown by the blocker-treated males during the breeding season. Kim interpreted her findings in light of the accumulating evidence for testosterone promoting certain context-dependent changes in male song, such as the expression of the competitive value of an individual male, rendering the song an honest signal.

In Chapter 5, Kim broadens her focus to study the endocrine regulation of behavior among several stonechat populations distributed across different latitudes. In this chapter, she and her co-authors compare testosterone concentrations of temperate zone and tropical stonechat populations during the breeding season within a pace-of-life framework. Tropical bird species tend to follow a slower pace of life, as they tend to have higher adult survival rates, longer breeding seasons and smaller clutches than temperate zone birds. It has been suggested that a slower pace of life is associated with lower levels of testosterone during the breeding season, which is something Kim has tested in this study by collecting blood samples from several temperate zone and tropical stonechat populations at different stages during the breeding season. Contrary to theory and published findings on other species, males from temperate zone and tropical stonechat populations did not differ in testosterone concentrations during any phase of the breeding season. Kim evaluated these findings in the context of current theories on the physiological mechanisms underlying differences in the pace of life of avian species.

Staying with stonechats as a study species, Kim reports in Chapter 6 on the characterization of 28 microsatellite markers for *Saxicola* species. This is more of a technical report, presenting the results of a genetical technique that is a crucial requirement for her follow-up study presented in Chapter 7. In Chapter 6, she analyzed the polymorphisms among these 28 markers, their linkage with sex and their usefulness for studying genetic diversity within the stonechat species complex, including some endangered populations.

Chapter 7 constitutes the heart of Kim's thesis. Here she developed a unique, comprehensive and in-depth study on the role of divergent breeding schedules ('allochrony') as one potential mechanism promoting population divergence in tropical stonechat populations. In East Africa, stonechat populations from two mountain ranges, the Great Rift Valley and the Eastern Arc Mountains, although spatially separated by only a few hundred kilometers, breed almost half a year apart. By integrating in a unique way genetic, behavioral and spatial modeling tools Kim studied the genetic, song and morphological divergence among twelve stonechat populations located along these two mountain ridges. Kim could show that allochronic and spatially separated populations are genetically differentiated. Spatial modeling further suggested that these genetically divergent allochronic populations are spatially connected, that is there were no obvious geographical or environmental barriers that would limit gene flow. These findings suggest that allochrony may be a driver of genetic diversification and thus one mechanism underlying population divergence. Furthermore, genetic diversity matched song and morphological divergence, and females showed a preference for the vocal and visual traits displayed by synchronously breeding males.

These results indicate that sexual selection may further reinforce population differentiation and may be an additional driver of divergence and potentially speciation. These findings are novel and important, and are of high relevance for current evolutionary concepts regarding the mechanisms underlying speciation in a wide variety of species.

With this thesis, Kim has proven herself to be an expert on bird song studies, in particular its endocrine regulation, relevance as a sexual and competitive signal, its possible role in population divergence and potentially even in speciation. In her thesis, she has successfully integrated divergent scientific approaches including behavioral, genetical, morphological, endocrinological, life history and spatial techniques to shed light on the evolutionary processes that generate population differences. I am certain that Kim's unique work will have a major impact within the field of biology, likely leading to a refinement of current evolutionary theories.

On the basis of the very high quality of the thesis, the many publications and the expected impact for the field, my grade for Kim Mortega's thesis will be 1.0 ('sehr gut').

Sincerely,

Michaela Hau

*Prof. Dr. Michaela Hau*
*Max Planck Institute for Ornithology, Seewiesen, Germany | Department of Biology, University of Konstanz*

University of Glasgow | Institute of Biodiversity, Animal Health & Comparative Medicine

# Gutachten

zur Dissertation von Kim Mortega zum Thema:

**„The contributions of seasonality, song and hormonal regulation to the evolution of songbirds“**

Kim Mortega befasst sich in ihrer Dissertation mit saisonalen Verhaltensweisen von Vögeln, mit besonderer Betonung auf Gesang. Sie interessiert sich dabei für mechanistische Grundlagen, ökologischen Kontext, und mögliche evolutionäre Konsequenzen. Mortega nutzt dazu eine breite Vielfalt an Techniken, von Gesangs- und Verhaltensanalysen über Hormonmessung zur genetischen Charakterisierung und zu komplizierten statistischen Analysemethoden. Das Projekt wurde in erster Linie von der DFG gefördert auf der Basis eines Drittmittelantrags, an dessen Erstellung Mortega wesentlich beteiligt war, und des Weiteren von der IMPRS.

Die Breite von Mortegas Ansatz verdient besondere Hervorhebung. In meiner Eigenschaft als Primärbetreuerin möchte ich ebenso betonen, dass Mortegas Dissertation aus zwei Gründen unter relativ schwierigen Bedingungen entstanden ist:

Da die Arbeit allochron brütender Vögel in Afrika vergleicht, war Mortega konfrontiert mit fast permanenter Brutaktivität, und dies fand statt in Ostafrika. Dieser Herausforderung ist Mortega zum Einen begegnet durch drei lange Afrikaexpeditionen, die insgesamt fast ein Jahr in Anspruch nahmen, und durch exzellente Koordination eines ausgedehnten, lokalen Freilandteams, das an verschiedenen Orten Daten für sie sammelte.

Kurz nach der Bewilligung des Projektes nahm ich eine Dauerstelle an der Universität Glasgow an, so dass der anfangs geplante enge Anschluss an die Universität Konstanz und das MPI für Ornithologie wegfiel, und die Betreuung ortsfern angesiedelt war. Mortega begegnete dieser zweiten Herausforderung ist Mortega durch lange Aufenthalte in Glasgow, und durch flexible Aufnahme von starken Themen im Vereinigten Königreich, insbesondere elaborierte genetische Studien und fortgeschrittene räumliche Statistik.

Trotz dieser Schwierigkeiten hat Mortega eine starke Dissertation mit insgesamt 7 Kapiteln verfasst. Diese

Kapitel spiegeln u.a. die breiten Kollaborationen von Frau Mortega, die jedoch noch nicht einmal vollständig repräsentiert sind: Mortega hat darüberhinaus auch Gesang für ein Meisenprojekt und Blutproben für ein Nachtigallenprojekt analysiert, die in der Dissertation nicht erwähnt sind.

Die Kapitel entwickeln sich anhand einer Linie, die mit Verhaltenstests zur örtlich differenzierten Gesangswahrnehmung von Schwarzkehlchen beginnt, und sich dann mit hormonalen und neuroendokrinen Mechanismen bei Schwarzkehlchen und Hausrotschwänzen auseinandersetzt, und schließlich genetische Muster im Detail analysiert. Die Gesangsanalysen sind bereits gut publiziert. Drei der Hormonarbeiten, die in Zusammenarbeit mit Wolfgang Goymann und Beate Apfelbeck entstanden, sind ebenfalls publiziert. Mortega hat zu diesen Kapiteln wesentliche Beiträge geleistet und vor allem ihre Gesangsexpertise bereichernd eingebracht. Daneben entsteht gerade auch eine endokrine Schwarzkehlchenpublikation, die aber noch in Anfangsstadien steht.

Der letzte Teil der Arbeit, die genetischen Analysen in Verbindung mit Ökologie und Verhalten, stellt den eigentlichen, und von der DFG geförderten Kern von Mortegas Dissertation dar. Hierin gingen die Felddaten der Afrikaexpeditionen ein, die genetischen Analysen in Sheffield, und die statistischen Raffinessen aus Glasgow, stark unterstützt durch Marielle van Tor in Radolfzell. Das Kapitel ist eine kraftvolle Verbindung von sehr diversen Ansätzen, die von Mortega vorangetrieben wurden. Das Kapitel allein steht bereits für eine insgesamt gelungene, breit angelegte Doktorarbeit.

Insgesamt vergebe ich die Note:

**magna cum laude – sehr gut: (1.0)**

Barbara Helm Glasgow, 21. Mai 2015

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# TABLE OF CONTENTS

## PART 1: Contribution of song to geographic differentiation

## CHAPTER 1 BEHAVIORAL RESPONSE OF A MIGRATORY SONGBIRD TO GEOGRAPHIC VARIATION IN SONG AND MORPHOLOGY

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# PART 2: Seasonal endocrine regulation of song and behavior

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Apfelbeck B, Mortega KG, Kiefer S, Kipper S, Michiel Vellema M, Villavicencio CP, Gahr M, Goymann W. 2013 General and Comparative Endocrinology 184 93–102

## CHAPTER 3 LIFE-HISTORY AND HORMONAL CONTROL OF AGGRESSION IN BLACK REDSTARTS: BLOCKING TESTOSTERONE DOES NOT DECREASE TERRITORIAL AGGRESSION, BUT CHANGES THE EMPHASIS OF VOCAL BEHAVIORS DURING SIMULATED TERRITORIAL INTRUSIONS

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* Joint first author

## PART 3: Evolutionary implications of divergence in genes, song, and seasonality

## CHAPTER 6 CHARACTERIZATION OF MICROSATELLITE MARKERS FOR *SAXICOLA* SPECIES

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## CHAPTER 7 ALLOCHRONIC AND GEOGRAPHIC REPRODUCTIVE ISOLATION IN AN AFRICAN SONGBIRD

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# Summary

Speciation by natural selection involves reproductive isolation between populations. In particular, ecological speciation, the divergence by local adaptation to different environments, has become recognized as an important evolutionary mechanism. It has been shown that even relatively weak selection for local adaptation can lead to strong genetic population structure, especially if it is reinforced by sexual selection. In this thesis, I investigate the relative contributions of seasonality, song and landscape to the genetic population structure, while also examining the role of hormones for seasonal variation in song. This question was addressed in tropical and temperate songbirds based on comprehensive field expeditions including experimental testing, combined with phenotypic, endocrine and genetic analyses, as well as sophisticated landscape genetics.

The first part of my thesis focuses on phenotypic drift between populations and their role for diversification with an emphasis on sexually selected traits of European stonechats, *Saxicola torquata rubicola*. Sexually selected traits contribute substantially to evolutionary diversification. In birds, sexually selected signals, in particular song and morphology, often undergo geographic divergence. In a first step, I assessed the geographic patterns of song and morphology traits in different Stonechat populations. I then conducted playback and decoy experiments to test their behavioral response to geographic variation in song and morphology. Females and males discriminated consistently between phenotypic traits from different populations of distinct geographic origin, responding stronger to local than to foreign stimuli. Furthermore, the behavioral response was consistently stronger for song than for morphology traits. I conclude that variation in sexually selected traits may contribute to geographic isolation over relatively short distances, and thereby promote local adaptation. This is remarkable since migratory birds are typically under time pressure. Migrants, for example, may have no choice but to pair with a less advantageous mate and their young may have no time to learn the local song accurately, which may counteract population divergence.

In the second part of my thesis, I investigated the role of endocrine regulations for the seasonal expression of song. A prominent life history trade-off involves the cost of reproduction and the cost of survival. This trade-off is proposed to be mediated by testosterone. Seasonal peaks in testosterone typically coincide with periods of intense competition between males for territories and mating partners. Interestingly, some temperate zone species also express territorial behavior outside a breeding context when testosterone levels are low. The Black redstart, *Phoenicurus ochruros*, defends territories not only during breeding, but also during the non-breeding season in fall. It is thus suitable to investigate the associations of seasonality, song and the endocrine regulation of territoriality. I compared spontaneous song during different life cycle stages, and experimentally blocked the action of testosterone and its estrogenic metabolites. Treated and untreated males were challenged with a

simulated territorial intrusion to test their behavioral response during breeding and non-breeding. I emphasized song traits known to play a crucial role in signaling of competitive interactions. The results suggest that territorial behavior may be decoupled from testosterone or its metabolites. In Black redstarts, testosterone apparently regulates specific components of territorial behavior that are directly linked to sexual selection. During breeding, only control males enhanced their song during territorial intrusions. During non-breeding however, when testosterone levels are naturally low, males of both treatment groups sang similar to breeding males with blocked testosterone. These results imply that during breeding testosterone seems to facilitate context-dependent changes in song in Black redstarts. I conclude that the role of testosterone in underlying endocrine regulations of seasonal activities during distinct life cycle stages seem to differ fundamentally across species, and may strongly depend on the diverse local adaptations of avian life histories.

In the last chapter of part 2, I studied the diverse life histories of Stonechats along a pace of life axis. In contrast to environments of the northern hemisphere where temperature and humidity change drastically over the year, environmental conditions in tropical biomes are relatively stable year-round. In general, tropical birds live a slower pace of life than temperate ones, i.e. they get older but produce fewer offspring during a single breeding season. Testosterone is one physiological factor proposed to mediate the pace of life, because its seasonal profile parallels a latitudinal trend along the slow-fast continuum. Tropical birds tend to have lower testosterone levels than species breeding at higher latitudes. My findings differed from the general endocrine pattern along a pace of life axis. Testosterone was elevated during breeding in both tropical and temperate stonechats. Furthermore, tropical stonechats responded as vigorously to a simulated territorial intruder as temperate stonechats with the strongest response during nestbuilding when testosterone levels were highest and females fertile. In addition, tropical males increased the peak song frequency during nestbuilding. Although migratory temperate stonechats sang with a higher song rate than resident tropical stonechats during mating, they did not have higher testosterone levels during that period. In contrast to other studied tropical birds, the breeding season of African stonechats is rather short. I suggest that in seasonally breeding tropical species, testosterone activates territorial behavior and facilitates mate guarding during the fertile period of females similar to temperate species. In contrast to other studies, testosterone does not seem to mediate differences in pace of life between tropical and temperate Stonechat species.

The final part added an evolutionary perspective. In twelve populations of the African stonechat, *Saxicola torquata*, I tested for relative contributions of allochronic, spatial and behavioral isolation leading to population divergence. In particular, I focus on the evolutionary dynamics of allochronic isolation, which remains mostly unconsidered when studying speciation. Populations might exhibit temporal barriers to gene flow, because seasonal activities must be accurately timed to avoid mismatches with the environment leading to severe fitness consequences. The evolution of rigid

reproductive schedules, endogenously generated biological rhythms, buffers organisms from responding to misleading, unpredictable variability of environments. The African stonechat breeds seasonally with pronounced reproductive schedules and asynchronous breeding onsets providing high potential for allochronic isolation. I examined the environmental connectivity between populations to quantify the relative temporal and spatial contributions to reproductive isolation. We found that allochronic populations of the Great Rift Valley and geographically separated populations from the Eastern Arc Mountains are genetically differentiated as judged from microsatellite loci and mitochondrial DNA. Genetically distinct populations of the Great Rift Valley have no geographic or spatial isolation barriers to gene flow, which strongly indicates population divergence as the result of allochronic isolation. However, this allochronic diversification may have occurred rather recently compared to the more ancient geographic diversification between populations of the two mountain ranges. The genetic structure is strongly concordant with patterns of song and morphology divergence. Furthermore, females prefer mates with synchronized reproductive timing, thus promoting reproductive barriers by behavioral isolation. The results indicate that the evolutionary dynamics of allochronic isolation and the ecological forces that shape them may be key drivers for population divergence and ultimately speciation.

In conclusion, the presence or absence of seasonality fundamentally shapes life history traits in Stonechats. In particular, the seasonal adaptation of song plays a major role in the formation of behavioral isolation, thus reinforcing population divergence even in the absence of spatial heterogeneity. The underlying regulation by testosterone seems to be especially crucial for seasonally breeding birds of temperate and tropical biomes. Understanding the diverse interactions of birds with their environments and their evolutionary implications will be important for assessing whether populations can adapt their physiology, behaviors and life histories fast enough to cope with the rapid climate change we are facing today.

# Zusammenfassung

Artbildung durch natürliche Selektion gründet auf dem Mechanismus der reproduktiven Isolation von Populationen. Insbesondere die ökologische Artbildung, als Varianz ortsgebundener Adaptationen an die gegebenen Umweltbedingungen, ist als zentrale evolutionäre Kraft erkannt worden. Es konnte gezeigt werden, dass selbst schwache Selektionsdrucke auf ortsgebundene Adaptationen einen starken Einfluss auf den Genpool einer Population haben können, insbesondere wenn diese durch die sexuelle Selektion verstärkt werden. In der vorliegenden Doktorarbeit werden die Einflüsse von Saisonalität, Gesang und Umwelt auf den Genpool einer Population, sowie die Rolle

der Hormone auf saisonale Gesangsvariationen untersucht. Diese Fragestellungen wurden mit umfassenden Feldstudien an Singvögeln in tropischen und gemäßigten Klimazonen bearbeitet. Die Feldstudien umfassten experimentelle Ansätze kombiniert mit phänotypischen, endokrinen und genetischen Analysen, sowie Habitatsanalysen.

Der erste Teil der Arbeit fokussiert auf die Bedeutung der phänotypischen Drift zwischen Populationen für die Diversifikation, mit dem Hauptaugenmerk auf den sexuell selektierten Merkmalen des Europäischen Schwarzkehlchens, *Saxicola torquata rubicola*. Sexuell selektierte Merkmale tragen wesentlich zur evolutionären Diversifikation bei. Diese sexuell selektierten Signale von Vögeln, insbesondere Gesang und Morphologie, unterliegen häufig geografischer Divergenz. Zunächst wurden geografische Muster in Gesängen und morphologischen Merkmalen in verschiedenen Populationen von Schwarzkelchen untersucht. In darauffolgenden Experimenten wurde das Antwortverhalten auf geografische Varianten von Gesang und morphologischen Merkmalen anhand von Playbacks und Attrappen untersucht. Sowohl Weibchen als auch Männchen unterschieden beständig zwischen den populationsspezifischen Phänotypen, indem sie eine stärkere Reaktion auf lokale als auf fremde Stimuli zeigten. Darüber hinaus beobachtete ich ein durchgehend stärkeres Antwortverhalten auf Gesang als auf morphologische Merkmale. Es lässt sich schlussfolgern, dass die Variation in sexuell selektierten Merkmalen zur geografischen Isolation über relativ kurze Distanzen beiträgt und somit lokale Adaptationen vorantreibt. Angesichts des Zeitdrucks, unter dem Zugvögel typischerweise stehen, ist dies bemerkenswert. Zugvögel könnten beispielsweise gezwungen sein, sich mit einem weniger vorteilhaften Partner zu paaren und ihre Jungen könnten aus Zeitmangel am Erlernen einer akkuraten lokalen Gesangsvariation gehindert werden, was der Varianz in der Population entgegenwirken würde.

Im zweiten Teil meiner Arbeit wurde die Rolle der endokrinen Regulation auf die saisonale Gesangsvariation untersucht. Ein herausragender Konflikt zwischen gegenläufiger Abhängigkeit, auch als Trade-off bezeichnet, umfasst die Kosten für Reproduktion gegenüber den Überlebenskosten. Es wird angenommen, dass dieser Trade-off durch Testosteron gesteuert wird. Saisonale Testosteron-Höchstwerte stimmen zeitlich mit saisonal ausgeprägter Konkurrenz um Territorien und Paarungspartnern überein. Interessanterweise zeigen in einigen gemäßigten Klimazonen einige Singvogelarten territoriales Verhalten auch außerhalb der Brutzeit, wenn der Testosteron-Spiegel niedrig ist. Der Hausrotschwanz, *Phoenicurus ochruros*, verteidigt sein Territorium nicht nur während der Brutzeit, sondern auch im Herbst, wenn diese Art nicht brütet. Daher ist diese Art dazu geeignet, die Verknüpfung von Saisonalität, Gesang und endokriner Regulation von Territorien zu untersuchen. In der vorliegenden Arbeit wurde spontaner Gesang während verschiedener Lebensphasen verglichen, und die Wirkung von Testosteron und seinem östrogenen

Stoffwechselprodukt experimentell blockiert. Männchen der Versuchs- und der Kontrollgruppe (ohne Hormonblockade) wurden mit einer simulierten territorialen Invasion während und außerhalb der Brutzeit auf ihre Verhaltensantwort getestet.

Die Ergebnisse deuten an, dass Territorialverhalten von der hormonellen Wirkung des Testosteron oder seiner Stoffwechselprodukte entkoppelt sein könnte. Offensichtlich reguliert Testosteron beim Hausrotschwanz spezifische Komponenten des territorialen Verhaltens, die direkt mit sexueller Selektion verknüpft sind. Während der Brutzeit haben nur die Männchen der Kontrollgruppe ihren Gesang während territorialer Invasionen erhöht. Außerhalb der Brutzeit, wenn die Testosteronwerte natürlicherweise gering sind, zeigten sowohl Männchen der Kontroll- als auch der Versuchsgruppe hingegen einen ähnlichen Gesang wie die Männchen der Versuchsgruppe während der Brutzeit. Dieses Ergebnis impliziert, dass Testosteron in der Brutzeit kontextabhängige Veränderungen im Gesang des Hausrotschwanzes bewirkt.

Es lässt sich schlussfolgern, dass sich die Funktionsweise von Testosteron in grundlegenden endokrinen Regulationsmechanismen der saisonalen Aktivität zwischen Singvogelarten wesentlich unterscheidet. Sie könnte stark von unterschiedlichen lokalen Adaptationen der Vögel, entsprechend ihrer Lebensumstände (life histories), abhängen.

Im letzten Kapitel des zweiten Teils untersuchte ich Testosteron während eines Abschnitts der Lebenszyklen von Schwarzkehlchen. Im Gegensatz zur nördlichen Hemisphäre, in der sich Temperatur und Feuchtigkeit im Jahresverlauf extrem ändern, sind die klimatischen Bedingungen in tropischen Lebensräumen das ganze Jahr über relativ stabil. Allgemein kann man sagen, dass tropische Vögel daher einen langsameren Lebensrhythmus haben als Vögel in der Gemäßigten Zone, d.h. sie werden älter, haben jedoch weniger Junge in einer Brutsaison. Man nimmt an, dass Testosteron ein physiologischer Faktor ist, der das Tempo des Lebensrhythmus regelt, weil saisonale Testosteronprofile latitudinale Gradienten entlang eines Langsam-Schnell-Kontinuums zeigen. Tropische Vögel haben tendenziell geringere Testosteronkonzentrationen als Arten, die in höheren Breiten brüten.

Meine Ergebnisse wichen von diesem allgemeinen Hormonlevel-Lebensrhythmus-Mustern ab. Testosteron war sowohl bei den tropischen als auch bei den in der gemäßigten Zone lebenden Schwarzkehlchen erhöht. Außerdem reagierten beide Gruppen von Schwarzkehlchen gleich stark auf einen simulierten territorialen Eindringling. Die stärkste Reaktion fiel in die Nestbau-Phase, wenn Testosteronkonzentrationen am höchsten und die Weibchen fruchtbar sind. Zusätzlich erhöhten tropische Vögel die Maximalfrequenz ihrer Gesänge während der Nestbauphase. Obwohl die in der gemäßigten Zone lebenden, ziehenden Schwarzkehlchen während der Paarungszeit mit höheren Gesangraten sangen als nicht-ziehende tropische Schwarzkehlchen, hatten sie keine höheren Testosteronkonzentrationen. Im Gegensatz zu anderen untersuchten tropischen Vögeln ist die

Brutsaison der afrikanischen Schwarzkehlchen relativ kurz. Vermutlich löst Testosteron bei saisonal brütenden tropischen Vögeln Territorialverhalten aus und reguliert Mate guarding während der fruchtbaren Phase der Weibchen – ähnlich wie bei Arten der Gemäßigten Zone. Im Gegensatz zu anderen Studien bewirkt Testosteron bei Schwarzkehlchen in tropischen und gemäßigten Zonen vermeintlich keine Unterschiede im Lebensrhythmus.

Im letzten Teil erweiterte ich meine Arbeit um eine evolutive Perspektive. An zwölf Populationen afrikanischer Schwarzkehlchen, *Saxicola torquata axillaris*, testete ich auf relative Einflüsse von allochroner und räumlicher Isolierung, sowie auf Isolierung aufgrund von Verhaltensunterschieden, die zur Isolierung von Populationen führen könnten. Im Speziellen konzentrierte ich mich auf die evolutionären Dynamiken durch allochrone Isolation, die bei der Erforschung von Artbildung meist unbeachtet bleibt. Populationen weisen oft zeitliche Limitierungen im Genfluss auf, weil ihre saisonalen Aktivitäten zeitlich sehr genau abgestimmt auf saisonale Veränderungen in der Umwelt sein müssen. Unstimmigkeiten würden schwere negative Fitnessfolgen nach sich ziehen. Die Evolution von rigiden Reproduktionszyklen auf der Basis endogen geregelter biologischer Rhythmen hilft den Organismen, zwischen richtigen Zeiten und irreführender oder unvorhersehbarer Umweltvariabilität zu unterscheiden.

Das afrikanische Schwarzkehlchen brütet saisonal und zeigte einen ausgeprägten zeitlich festgelegten Reproduktionsablauf, die eine potentielle Grundlage für allochrone Isolation bietet. Ich untersuchte die räumliche Verknüpfung zwischen den Populationen, um den relativen zeitlichen und räumlichen Einfluss auf die reproduktive Isolation zu quantifizieren. Wir fanden heraus, dass sich die allochronen Populationen des Great Rift Valley und die davon geographisch getrennten Populationen der Eastern Arc Mountains genetisch unterscheiden. Dieser Befund wurde durch Mikrosatelliten und mitochondriale DNA ermittelt. Genetisch voneinander unterschiedliche Populationen des Great Rift Valley zeigten keine geographische Isolation des Genflusses, was stark dafür spricht, dass die Populationsunterschiede ein Ergebnis der allochronen Isolation darstellen. Diese allochrone Diversifikation könnte später entstanden sein als die frühe geographische Diversifikation der Populationen durch die zwei Gebirgsketten Die genetischen Befunde befinden sich im Einklang mit den jeweiligen Unterschieden in Gesang und Morphologie. Außerdem bevorzugen Weibchen Stimuli von Männchen aus synchronisierten Populationen, was für Isolationsmechanimsmen durch Verhalten spricht. Die Ergebnisse zeigen, dass evolutionäre Dynamiken der allochronen Isolation und die ökologischen Gegebenheiten, die diese ausbilden, Schlüsselfaktoren für Populationsunterschiede und letztendlich für die Artenbildung sein können.

Zusammenfassend kann man sagen, dass Saisonalität grundlegend die Lebensumstände (life history) und die Ausprägung von lokalen Anpassungen von Schwarzkehlchen bestimmt. Besonders die saisonale Anpassung des Gesangs spielt eine große Rolle beim Ausbilden von Verhaltens-Isolation. Dadurch wird die Divergenz von Populationen sogar ohne räumliche Trennung verstärkt. Die zugrundeliegende Regulation durch Testosteron scheint besonders entscheidend für saisonal brütende Vögel der gemäßigten und der tropischen Zone zu sein. Das Verständnis dieser verschiedenen Interaktionen der Vögel mit ihrer Umgebung und ihrer evolutiven Auswirkungen ist entscheidend für eine Einschätzung der Fähigkeit von Populationen, in angemessener Weise ihre Physiologie, Verhalten und Lebenszyklus anzupassen, um mit den aktuellen rapiden Umweltveränderungen Schritt halten zu können.

# General Introduction

The evolution of reproductive isolation between populations by divergent natural selection commonly drives the origin of species (Schluter 2001). Many studies attempt to infer evolutionary processes from current differences in genetic, vocal, morphological, ecological, and behavioral traits (Coyne and Orr 2004). In an integrative approach, this study aims to determine the relative contributions and interplay of all these traits leading to population divergence, thus contributing to the development of our understanding of evolution. In particular, I focus on ecological speciation, the divergence by local adaptation to different environments in birds. Within the concept of ecological speciation, the local adaptation hypothesis predicts that birds which select mates from their natal regions will gain fitness advantages because their offspring will more likely express adaptations to local ecological conditions (Kawecki and Ebert 2004). Benefits of local adaptation contrast with potential disadvantages such as inbreeding, which is generally avoided through dispersal. According to the local adaptation hypothesis, these disadvantages are outweighed by the benefits of selecting local mates. However, although ecological conditions may induce natural selection for local adaptation, disruptive or diversifying selection is generally not sufficient for speciation if individuals can migrate between populations. In this case, a mating system has to emerge that strongly reduces gene flow between ecologically diverging populations (Coyne and Orr 2004).

In this thesis, I am particularly interested in natural and sexual selection related to the role of seasonality, and in behavior (song) and physiology (hormones) that play important roles in adaptation to seasonal environments (Helm et al. 2009; Baldwin et al. 2010).

## Song

Surprisingly, only very little is known about whether and how interactions of natural and sexual selection facilitate or constrain speciation (reviewed in (Maan and Seehausen 2011)). Biologists often either consider sexual selection in isolation from natural selection, or address ecological effects on sexually selected traits, but rarely assess the implications for population divergence. In this study, I aim to integrate several components of potential speciation-facilitating mechanisms focusing on both, natural and sexual selection with emphasis on the effects of seasonality and song, respectively, and their interactions on population divergence. Hereby, I make inferences about the dynamics of genetic differentiation using sophisticated statistical landscape genetics. Adaptive traits favored by environmental factors may support diversification that emerges from interactions between sexual selection and environmental heterogeneity (reviewed in (Maan and Seehausen 2011)). Phenotypic traits involved in signaling, for example aspects of song and morphology, have been identified to contribute to reproductive isolation between diverging populations (Marler 1957; Coyne and Orr 2004). Specifically, signaling in the context of resource defense or mate attraction may enhance reproductive isolation through assortative mating (Grant and Grant 2002; Edwards et al. 2005; Price and Sol 2008; Podos 2010). In many songbirds, songs are a key component of signaling and are transmitted across generations via vocal learning (Slabbekoorn and Smith 2002). Accordingly, song dialects, i.e. the unique repertoire of shared songs within a population, combined with female preference for a local dialect due to parental imprinting, may lead to reproductive divergence (Marler and Tamura 1962; Nottebohm 1969; Baker 1975; Searcy 1992).

The relationship between local adaptation and associated signaling on the one hand, and female preference on the other, can also be affected by a species' life history (Helbig 2003). In particular, avian migration may counteract reproductive divergence (Paradis et al. 1998; Nelson 1999; Helbig 2003) because migrants are typically under time pressure to make rapid reproductive decisions (Reed et al. 1999), and may therefore not pair with the most advantageous, locally adapted mate available (Randler 2002). Moreover, in migratory birds, individuals of higher phenotypic quality are often observed to arrive and mate first (Francis and Cooke 1988; Lundberg and Alatalo 1992; Møller 1994; Lozano et al. 1995). This phenomenon is commonly explained by the reasoning that birds with advanced seasonal reproductive timing may gain the best territories, but only high quality males will be able to survive the costs associated with early arrival (Kokko 1999).

## Seasonality

Successful breeding requires that birds closely match their breeding period, and thus their reproductive behavior with the often brief moment when environmental conditions are favorable (Prestt 1971; Murton and Westwood 1977). Temporal shifts in life history stages towards favorable environmental conditions, for example through increased abundance of resources, are thus likely to foster survival and reproductive success (Durant et al. 2007). Under these circumstances, the adaptation to diversifying seasonal environments may promote such phenotypic divergence between populations even more rapidly. Because seasonal activities must be accurately timed to avoid mismatches with the environment (Bradshaw and Holzapfel 2007), some animals have rigid circannual rhythms, i.e. endogenously generated biological rhythms of approximately one year. This has the advantage of regulating timing under a suite of conditions and of buffering organisms from responding to misleading, random environmental variability (Hahn et al. 1997). The timing of reproduction is considered as one of the major life history traits reflecting the adaptation of birds to local features of their environment (Visser and Lambrechts 1999). This pattern of local adaptation to the seasonality of the environment has striking implications on a larger geographical scale. Organisms tend to cluster along a latitudinal gradient associated with differences in their life histories, the pace of life axis. In the northern hemisphere, temperate zone birds are relatively short-lived and produce a high number of offspring, which develop relatively fast and mature early in life, the fast end of the life-history axis, whereas tropical birds lie at the opposite end of this continuum (Wiersma et al. 2007).

A major aim of this study is to understand the contributions of these adaptive temporal patterns in avian life histories to evolution. In addition to environmental seasonality and song, this includes an interest in the underlying endocrine regulations.

## Hormones

The physiological and behavioral mechanisms that lead to life history trade-offs are often under endocrine regulation (Stearns 1989). Testosterone is one physiological factor proposed to mediate the trade-off between reproduction and survival (reviewed in (Hau 2007)). In particular, it has important pleiotropic effects on resource allocation for mate attraction, competitive behavior, reproductive effort, and associated signal plasticity, while often simultaneously decreasing fitness by suppressing traits such as immune function and parental care. Therefore, seasonal peaks in testosterone can be brief and typically coincide with periods of intense competition between males for territories and mating partners. This study aims to determine the time patterns of testosterone production in a seasonally changing environment, and the diverse effects it has on song, physiology and behavior.

Environmentally induced phenotypic variation allows developing organisms to respond adaptively to changes in their environment, and can directly alter an individual's behavior, morphology and physiology. On a large scale, this phenotypic variation in testosterone levels seems to follow a latitudinal trend along the pace of life axis. Tropical birds tend to have lower testosterone levels (Garamszegi et al. 2008) and seem to lack a peak in testosterone at the beginning of the breeding season (Wikelski et al. 2003a; Onofrei et al. 2004) compared to species breeding at higher latitudes. In a comparative approach along the pace of life axis, I aim to test if testosterone may act as a proximate mediator of latitudinal phenotypic and behavioral variation within the Stonechat complex, a study species with well-known life history traits. For a more detailed examination of the associations of seasonality, song and the hormonal regulation in the context of territoriality, I studied the Black redstarts, *Phoenicurus ochruros,* during distinct life cycle stages.

## Study species

To address all the questions I have introduced in the previous paragraphs, I studied the Stonechat complex, *Saxicola torquata*. In stonechats, an important mechanism, which may have promoted the diverse life histories with pronounced reproductive schedules, is their endogenous, circannual breeding rhythm. They have developed a suite of traits that allow them to master the seasonality of their respective breeding grounds. Interestingly, resident populations of African stonechats, *Saxicola torquata axillaris*, have adjusted their reproductive timing to diversifying microclimatic local differences in the environment, which may imply a formation of temporal barriers to gene flow. As reported for other species, genetic diversity in Stonechats is particularly high in tropical populations (Jetz and Rahbek 2002; Illera et al. 2008; Woog et al. 2008). While the drivers of these differences have not been studied, the heterogeneous environmental seasonality over short distances in East Africa implies a possible role for allochrony, i.e. phenotypic and genotypic divergence of populations by timing. I set out to study potential geographic and, more importantly, allochronic reproductive isolation mechanisms in East African stonechats.

Stonechats occupy a wide geographical range; they not only live in afrotropical but also temperate environments, and are therefore ideal to address the endocrine basis for life history trade-offs on a pace of life axis. Previous studies suggest that 'specific life-history traits may play a more important role in determining testosterone profiles of tropical and non-tropical birds than breeding latitude and encourage further studies to allow for more formal comparisons' (Goymann and Landys 2011), and that 'additional social and life history variables may need to be considered if we aim at gaining an integrated understanding of the ultimate factors that influence the relationship between hormonal signals and behavioral traits in natural populations' (Hau et al. 2008). This present study aims to incorporate the assembled knowledge in Stonechats and advance the understanding of the evolutionary processes that are at the basis of variations in hormone–behavior interactions with a specific focus on song, which so far has been neglected in this context.

For a more detailed evaluation of the associations of seasonality, song and the hormonal regulation in the context of territoriality, I also studied the Black redstart, *Phoenicurus ochruros*. This species is a highly territorial songbird with well-known life cycle stages, and a remarkably active song period during the nonbreeding season in fall. It is thus an ideal study species to investigate the role of song in territorial behavior, and details of the interplay between testosterone, territorial aggression and signal plasticity. This is examined using phenotypic engineering by hormonal manipulations (Ketterson et al. 1996; Hau 2007).

The initial idea to bridge physiological and behavioral implications with an explicit focus on song during territorial disputes, arose from an extensive collaboration with my colleagues from the Max Planck Institute for Ornithology, Beate Apfelbeck and Wolfgang Goymann, and from the Free

University of Berlin, Sarah Kiefer and Silke Kipper. The integration of our expertise in bioacoustic communication enabled us to develop an integrative approach to the study of the endocrine regulation in Black redstarts at the Max Planck Institute. This complements the PhD project of Beate Apfelbeck and is included as three chapters of her dissertation at the Ludwig Maximilian University of Munich (2011). Together we have published these three chapters in peer-reviewed international journals in 2012 and 2013. Together with Beate Apfelbeck and Sarah Kiefer, I am a joint first author of one of these publications 'Testosterone affects song modulation during simulated territorial intrusions in male Black redstarts (*Phoenicurus ochruros*)'.

## PART 1: Contribution of song to geographic differentiation

In Chapter 1, I examine the relative contribution of song and morphology to geographic differentiation in the migratory European stonechat. For this, I first quantified the diversity of song traits from different populations. Diversifying phenotypic patterns may be used by both sexes in the context of sexual selection and territoriality. I then tested the responses of free-living Stonechats of both sexes to male phenotype with playbacks and decoys, representing local and foreign stimuli derived from a range of distances from the local population. The ways conspecifics respond to geographic variation in phenotypes can give insights to possible behavioral isolation barriers, but these may depend on migratory behavior. Earlier studies have reported lower song discrimination in migrant than resident species, but have also indicated mechanisms by which migrants could nonetheless learn local song dialects after dispersal (Nelson et al. 1996; Nelson 1999). Evaluating an individual's perception of geographic variation in sexually selected traits is a crucial first step for understanding reproductive isolation mechanisms.

## PART 2: Seasonal endocrine regulation of song and behavior

In part 2, I discuss the underlying endocrine regulations of adaptations of seasonal activities during distinct life cycle stages. The Black redstart is of particular interest in this regard, because the degree to which testosterone facilitates seasonal territorial behavior can be determined for reproductive and comparatively for non-reproductive life cycle stages. In this species, only those components of breeding territoriality that function simultaneously as sexually selected signals may be under control of sex steroids.

In the first three chapters of part 2, I explore the endocrine underpinnings of phenotypic variation in song incorporating brain receptor expression in a reproductive context. In vertebrates, the most conclusive experimental approach to investigate phenotypic variation and reproductive trade-offs may be phenotypic engineering, i.e. the manipulation of circulating hormone concentrations in individuals (Ketterson et al. 1996). I treated territorial males with an anti-androgen and an aromatase inhibitor during the breeding and non-breeding season to block both, the direct and indirect effects of testosterone. After the treatment, implanted males were challenged with a simulated territorial intrusion, and their behavioral response was documented. A highlight of these studies is the emphasis on various song traits associated with mate attraction and resource defense to test if testosterone acts specifically on signals that communicate the motivation or ability of individuals to engage in competitive situations. Context-dependent song during breeding may be facilitated by testosterone or potentially by conversion of testosterone to estradiol in the preoptic area of the brain.

In this study, I have examined the relationship between testosterone and individual traits in a temperate zone bird species. In the majority of male temperate zone vertebrates, circulating concentrations of testosterone increase during the breeding season to promote reproductive behavior. In birds, comparative studies documented that tropical species have lower peak testosterone concentrations during the breeding season compared to non-tropical species, and that seasonal fluctuations in testosterone can be slight or even absent (Dittami and Gwinner 1990; Wikelski et al. 2003a; Goymann et al. 2004). Recent studies, however, revealed that some tropical species with short breeding seasons express elevated testosterone levels during periods of maximum female fecundity (Goymann et al. 2004, 2006). Thus, the length of breeding season and additional environmental factors not directly related to latitude may foster conditions under which high testosterone levels are beneficial in the tropics (Rödl et al. 2004).

In the fourth chapter of part 2, I studied nine tropical and six temperate Stonechat populations along a latitudinal gradient to directly link latitudinal comparisons of endocrine regulations with temporal patterns in avian life histories. I compared testosterone levels during different breeding stages in males and assessed their behavioral response towards an intruder via playback experiments. I specifically emphasized on diversifying patterns of song in relation to latitudinal variation in testosterone, which so far have not been considered in this context, but which plays a major role in the communication of resource defense, mate attraction, and the protection of paternity.

## PART 3: Evolutionary implications of divergence in song, seasonality and landscape

The diverse temporal patterns in life histories may be an important driver for the outstanding diversity of the avian fauna. In this core part of my dissertation, I aim to explore the evolutionary implications of the divergence in song, seasonality, and landscape. Specifically, I focus on the evolutionary dynamics of allochronic isolation and the ecological forces that shape them, which may be key drivers for population divergence and ultimately speciation. The mechanisms that have driven allochrony, a possible involvement of biological rhythms, and the relative roles of spatial and temporal environment, are poorly understood. To address these questions, I studied African stonechats, songbirds representative of endogenous, circannual rhythmicity, with high genetic differentiation (Illera et al. 2008; Woog et al. 2008).

In the first chapter of part 3, I developed and characterized 28 novel microsatellite loci for African stonechat to determine the genetic divergence between study populations. All markers also successfully amplified in nine different *Saxicola* species ranging from Europe to Asia, including three endemic Island species. These microsatellite markers will not only be useful for this present study but also for the assessment of the genetic diversity of the large and widely distributed genus *Saxicola*.

In the final chapter, I set out to integrate the relative contributions of allochronic, spatial and behavioral isolation leading to population divergence. I compared the variation on 18 microsatellite loci and the mitochondrial cytochrome b among twelve African stonechat populations. The newly developed molecular tools in combination with existing rich field data and well-characterized habitat use of Stonechats facilitate parallel consideration of temporal and spatial aspects of the environment. Recent advances in landscape genetics allow a more precise understanding of the relative contributions of diverse environmental aspects to population divergence. Together with my expertise in the biology of African stonechats and the profound knowledge in spatial ecology modeling of my collaborator, Mariëlle van Toor from the Max Planck Institute for Ornithology, generalized functional responses for environmental data were estimated to describe the habitat available at the study area (Matthiopoulos et al. 2011). Mariëlle then estimated the habitat connectivity between populations and calculated pairwise resistances for all populations using the final habitat usage map as a resistance landscape (McRae et al. 2008). With the valuable advice from my two collaborators, Jason Matthiopoulos and Paul Johnson from the University of Glasgow, I then implemented sophisticated landscape genetics models to infer the relative contributions of temporal and spatial aspects leading to population divergence, thus contributing to the development of our understanding of evolution.

In an era of climate change, organisms are exposed to constant change in their environments. In order to maintain their fitness and survival, they require adapting to such environmental change reliably (Schluter 2001, 2009). Local adaptation to diverging environments thus may enhance adaptive divergence, rapid evolutionary dynamics, and ultimately speciation.

# PART 1: Contribution of song to geographic differentiation

# CHAPTER 1 Behavioral response of a migratory songbird to geographic variation in song and morphology

*Mortega KG, Flinks H, Helm B. 2014 Frontiers in Zoology 11:85*

# Abstract

### *Introduction*

Sexually selected traits contribute substantially to evolutionary diversification, for example by promoting assortative mating. The contributing traits and their relevance for reproductive isolation differ between species. In birds, sexually selected acoustic and visual signals often undergo geographic divergence. Clines in these phenotypes may be used by both sexes in the context of sexual selection and territoriality. The ways conspecifics respond to geographic variation in phenotypes can give insights to possible behavioral barriers, but these may depend on migratory behavior. We studied a migratory songbird, the Stonechat, and tested its responsiveness to geographic variation in male song and morphology. The traits are acquired differently, with possible implications for population divergence. Song can evolve quickly through cultural transmission, and thus may contribute more to the establishment of geographic variation than inherited morphological traits. We first quantified the diversity of song traits from different populations. We then tested the responses of free-living Stonechats of both sexes to male phenotype with playbacks and decoys, representing local and foreign stimuli derived from a range of distances from the local population.

### *Results*

Both sexes discriminated consistently between stimuli from different populations, responding more strongly to acoustic and morphological traits of local than foreign stimuli. Time to approach increased, and time spent close to the stimuli and number of tail flips decreased consistently with geographic distance of the stimulus from the local population. Discriminatory response behavior was more consistent for acoustic than for morphological traits. Song traits of the local population differed significantly from those of other populations.

### *Conclusions*

Evaluating an individual's perception of geographic variation in sexually selected traits is a crucial first step for understanding reproductive isolation mechanisms. We have demonstrated that in both sexes of Stonechats the responsiveness to acoustic and visual signals decreased with increasing geographic distance of stimulus origin. These findings confirm consistent, fine discrimination for both learned song and inherited morphological traits in these migratory birds. Maintenance or further divergence in phenotypic traits could lead to assortative mating, reproductive isolation, and potentially speciation.

# Introduction

Phenotypic traits involved in signaling, for example aspects of song and morphology, are known to contribute to reproductive isolation between diverging populations (Marler 1957; Coyne and Orr 2004). Specifically, signaling in the context of mate attraction or territoriality may promote reproductive isolation through assortative mating and settlement patterns (Edwards et al. 2005; Price and Sol 2008; Podos 2010). In birds, both sexes can be actively involved in signaling and also in discrimination of local conspecifics as potential sexual partners or sexual competitors (Grant and Grant 2002).

In most songbirds, songs are a key component of signaling and are culturally transmitted across generations via vocal learning (Slabbekoorn and Smith 2002). Young birds learn to produce or recognize song early in life, while still in their natal region. The geographic variation of such song traits is thought to result from the effect of imperfect song copying (Baker and Cunningham 1985). Accordingly, song dialects, i.e. the unique repertoire of shared songs within a population, combined with female preference for a local dialect due to parental imprinting, may lead to reproductive divergence (Marler and Tamura 1962; Nottebohm 1969; Baker 1975; Searcy 1992). Female preference for familiar vocalizations has been shown in some captive and field experiments by increased copulation-solicitation displays to standardized playback (Baker 1982, 1983; Searcy et al. 2002; Danner et al. 2011).

Often not only vocalizations but a suite of selected traits of different sensory modalities contribute to the establishment and maintenance of reproductive isolation (Uy et al. 2009a). For example, morphological traits are also proposed to facilitate pre-mating isolation barriers between related avian lineages (Seddon et al. 2013). Such traits often include plumage coloration, e.g. plumage redness in house finches, *Carpodacus mexicanus* (Mcgraw et al. 2001). In golden-collared manakins, *Manacus ssp.*, the golden is preferred over the white phenotype (Stein and Uy 2006). Genetically inherited visual signals may therefore facilitate diversification (Price 1998; Kirschel et al. 2009). In contrast, sexually selected traits that are inherited culturally, notably learned avian vocalizations, can change instantaneously without requiring genetic change. They may therefore be a more efficient mechanism for reproductive isolation than inherited traits (Baker and Mewaldt 1978; Patten et al. 2004; Grant and Grant 2009, 2012).

By promoting isolation, geographically differentiated signals are thought to aid local adaptation. The local adaptation hypothesis predicts that birds which select mates from their natal regions will gain fitness advantages because their offspring will more likely express adaptations to local ecological conditions (Kawecki and Ebert 2004), for example adaptations of seasonal activities associated with local climates, or morphologies tailored to specific lifestyles (Helm et al. 2009; Baldwin et al. 2010). For instance, there is evidence from North American crossbills (*Loxia curvirostra* - complex) for distinct song types associated with incipient speciation (Benkman 1993, 2003; Smith and Benkman 2007). Interestingly, the differences in song types are coupled with morphological differences relating to ecological speciation. However, the processes of local adaptation and associated signaling may be sensitive to movement behavior (Helbig 2003). Migration may counteract population divergence (Nelson 1999) because: a) migration is thought to correlate positively with dispersal distance, which in turn generally promotes gene exchange (Paradis et al. 1998; Helbig 2003); b) migrants are typically under pressure to make rapid reproductive decisions, implying that female migrants may be less choosy than female residents (Reed et al. 1999), and may therefore not pair with the best (i.e., locally adapted) mate available (Randler 2002); c) relating to acoustic signals, migratory departure after breeding limits opportunities for young males and females to learn or imprint to the local dialect. Earlier studies have reported lower song discrimination in migrant than resident species, but have also indicated mechanisms by which migrants could nonetheless learn local song dialects after dispersal (Nelson et al. 1996; Nelson 1999).

To better understand processes of local differentiation, in particular in migratory birds, we investigated discriminatory abilities in Stonechats (*Saxicola torquata* and closely related lineages (Illera et al. 2008)). The *Saxicola* complex has a wide distribution range, comprising substantial local differentiation in seasonal and morphological traits (Helm et al. 2006). We focused on the short-distance migrant European stonechat (*Saxicola torquata*), which is socially monogamous with seasonal pair bonds selected by females (Flinks et al. 2008). During the entire breeding season, males defend their territory with distinct behavioral responses. Females also actively respond to conspecific intruders (Canoine and Gwinner 2005). The fact that males sometimes "punish" their mates for their response to intruders indicates a sexual context to female interest (Canoine and Gwinner 2002). This responsiveness allowed us to examine discriminatory abilities in both sexes. We studied song variation between Stonechat populations and tested the behavioral response of the focal European population to song recordings and stuffed decoys. Early in the breeding season we obtained and analyzed song repertoires of the local population and additional populations that breed 90 and 180 km away. We experimentally tested the responsiveness of local Stonechats to song from these populations and to stimuli from African Stonechats and a control species by conducting simulated territorial intrusions with playbacks. We also conducted a decoy experiment simulating a territorial intrusion by presenting a taxidermic mount of phenotypes from populations with differing geographic distances. The experiments focused on male response, but we also report data on the latency of the female response to the stimuli. All experiments were conducted during the breeding season at defined breeding stages in the presence of both pair mates.

In view of the geographic differentiation within Stonechats, we hypothesized that despite their migratory behavior female and male Stonechats i) can discriminate between phenotypes of geographically distinct populations during playback and decoy experiments, ii) respond most strongly to local population stimuli, and iii) may show a consistent decline in their responsiveness with geographic distance. Furthermore, we hypothesized that songs may elicit stronger responses than morphological traits in both sexes because they may have diverged more rapidly.

# Results

## Song traits

The Stonechat populations differed in their song traits from each other. A principal component analysis of seven traits (Table 1.1a) showed that several principal components explained the variation in song (PC1 = 37.26, PC2 = 29.17, PC3 = 21.62, Fig. 1.1). Based on the first principal component, the focal population differed significantly from the neighboring population (90 km). Differences increased further with geographic distance from the local population (Additional file 1: Table S1, Fig. S1), although Stonechats from 90 km and 180 km were not significantly different from each other (Table 1b, Fig. 1.1).

Table 1.1 **Song traits**

| | **PC 1** | **PC 2** | **PC 3** | |
|---|---|---|---|---|
| (a) | | | | |
| no. of elements | 0.46 | 0.38 | 0.29 | |
| element rate | −0.27 | −0.29 | 0.46 | |
| peak frequency | −0.02 | 0.30 | −0.63 | |
| min. frequency | −0.23 | 0.61 | −0.04 | |
| max. frequency | 0.53 | −0.08 | −0.27 | |
| bandwidth | 0.47 | −0.44 | −0.14 | |
| eigenvalue | 2.61 | 2.04 | 1.51 | |
| % variance | 37.26 | 29.17 | 21.62 | |
| (b) | | | | |
| **fixed effects** | **estimate** | **s.e.m** | **t** | **p** |
| **intercept** | −1.76 | 0.12 | **−14.89** | **<0.001** |
| **90 km** | 2.28 | 0.29 | **7.97** | **<0.001** |
| **180 km** | 2.38 | 0.18 | **13.52** | **<0.001** |

|  | PC 1 | PC 2 | PC 3 |  |
|---|---|---|---|---|
| (a) |  |  |  |  |
| no. of elements | 0.46 | 0.38 | 0.29 |  |
| element rate | −0.27 | −0.29 | 0.46 |  |
| peak frequency | −0.02 | 0.30 | −0.63 |  |
| min. frequency | −0.23 | 0.61 | −0.04 |  |
| **African** | 0.64 | 0.19 | **3.44** | **<0.001** |
| **control** | 4.79 | 0.19 | **25.45** | **<0.001** |

(a) Factor loadings of the principal component analysis for seven song traits of European Stonechats from the local population, a population from 90 km distance, a population from 180 km distance, African stonechats, and the winter wren. (b) Results of general linear model testing whether the first principal component (PC1) differed between songs from different locations, estimated by maximum likelihood methods. Estimates for the different song locations refer to differences from the intercept estimate, which represents song traits of the local population. Subjects were included as random intercepts to control for repeated measures. ‘Significant’ differences are shown in bold.

## Playback and decoy experiments

Stonechats of the local population responded differently to stimuli from distinct populations, measured by the time they took to approach the caller or decoy (i.e., latency to approach within 5 m). In response to playback, males discriminated significantly between origins of the stimulus ($z = -8.42$, $p < 0.001$, Table 1.2a, Fig. 1.2). The males’ latency to approach the caller was lowest when exposed to the local song and increased with distance of stimulus origin (Table 1.2a, Fig. 1.2). Breeding stage, trial order (Additional file 1: Fig. S4), date and time of day showed no significant effect on the males’ latency to approach (Table 1.2a).

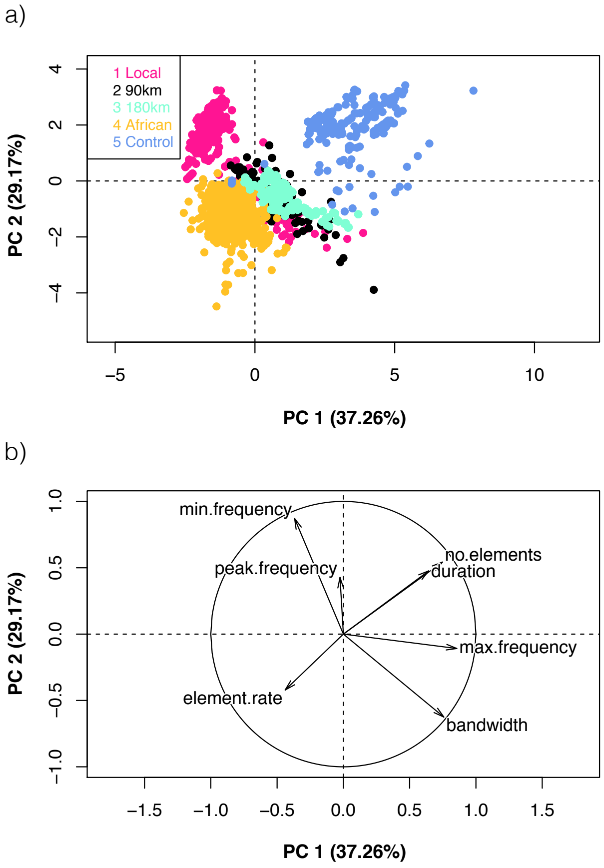

**Fig. 1.1 Geographic variation in the song of stonechats and a control species as quantified by principal component analysis.** Shown is (a) the variation in song structure of European Stonechats from (1) the local population, (2) a population from 90 km distance, (3) a population from 180 km distance, (4) African stonechats, and (5) control species (Winter wren) based on a principal component analysis (for details, see Table 1.1); and (b) factor loadings of the two first principle components for song duration, number of elements, element rate, the minimum, maximum and peak frequency, and the bandwidth. The arrow length indicates the degree, the arrow direction the association of factor loadings with the principal components PC 1 and PC 2.

**Table 1.2 Latency to approach within 5 m**

| | Fixed effects | Estimate | Hazard ratio | s.e.m | z | p |
|---|---|---|---|---|---|---|
| **playback** | | | | | | |
| (a) males | **origin** | −0.86 | 0.42 | 0.10 | −8.42 | **<0.001** |
| | **90 km** | −1.30 | 0.27 | 0.31 | −4.17 | **<0.001** |
| | **180 km** | −2.17 | 0.11 | 0.34 | −6.36 | **<0.001** |
| | **African** | −2.32 | 0.10 | 0.35 | −6.66 | **<0.001** |
| | **control** | −4.18 | 0.02 | 0.53 | −7.85 | **<0.001** |
| | breeding stage | 0.60 | 1.82 | 0.59 | 1.02 | 0.31 |
| | trial order | 0.04 | 1.04 | 0.07 | 0.52 | 0.61 |
| | date | 0.08 | 1.09 | 0.08 | 1.06 | 0.29 |
| | time | 0.004 | 1.00 | 0.03 | 0.16 | 0.88 |
| (b) females | **origin** | −1.36 | 0.26 | 0.22 | −6.28 | **<0.001** |
| | **90 km** | −2.11 | 0.12 | 0.49 | −4.32 | **<0.001** |
| | **180 km** | −2.40 | 0.09 | 0.54 | −4.45 | **<0.001** |
| | **African** | −4.51 | 0.01 | 0.85 | −5.32 | **<0.001** |
| | **control** | −6.88 | 0.009 | 0.97 | −7.84 | **<0.001** |
| | breeding stage | −0.75 | 0.47 | 0.41 | −1.84 | 0.06 |
| | trial order | 0.16 | 1.18 | 1.27 | 1.29 | 0.20 |
| | date | 0.02 | 1.02 | 0.07 | 0.23 | 0.82 |
| | time | −0.09 | 0.91 | 0.06 | −1.60 | 0.11 |
| **decoy** | | | | | | |
| (c) males | **origin** | −0.78 | 0.46 | 0.16 | −4.93 | **<0.001** |
| | European | −0.43 | 0.65 | 0.41 | −1.03 | 0.30 |
| | African | −0.65 | 0.52 | 0.43 | −1.52 | 0.13 |
| | **Control** | −2.95 | 0.05 | 0.64 | −4.62 | **<0.001** |
| | breeding stage | 0.08 | 1.08 | 0.17 | 0.46 | 0.65 |
| | trial order | −0.18 | 0.83 | 0.16 | −1.18 | 0.24 |
| | date | −0.05 | 0.95 | 0.11 | −0.49 | 0.62 |
| | time | −0.001 | 0.99 | 0.009 | −0.19 | 0.85 |
| (d) females | **origin** | −0.79 | 0.45 | 0.16 | −4.84 | **<0.001** |
| | **European** | −1.50 | 0.22 | 0.56 | −2.65 | **0.007** |
| | **African** | −1.70 | 0.18 | 0.59 | −2.87 | **0.004** |
| | **control** | −3.19 | 0.04 | 0.84 | −3.78 | **<0.001** |
| | breeding stage | −0.10 | 0.10 | 0.18 | −0.54 | 0.59 |
| | trial order | −0.10 | 0.10 | 0.16 | −0.59 | 0.55 |
| | date | −0.15 | 0.86 | 0.10 | −1.44 | 0.15 |
| | time | −0.01 | 0.99 | 0.009 | −1.50 | 0.13 |

Results of cox mixed-effects model with estimates, hazard ratio, standard error, z-value, and p-value fitted by maximum likelihood for playback in (a) males and (b) females, and decoy experiment in (c) males and (d) females. Estimates refer to differences from the intercept estimate, which represents the latency to approach of the local population (not shown). 'Origin' represents the overall estimate of differences between populations. 'Significant' differences are shown in bold

Likewise, females also differed significantly in their behavioral response to different playback stimuli ($z = -6.28$ $p < 0.001$, Table 1.2b, Fig. 1.3). The females' latency to approach the caller was lowest when presented with the local song and increased with geographic distance of the stimulus origin (Table 1.2b, Fig. 1.3). Breeding stage, trial order, date and time had no significant effects on the females' latency to approach the caller (Table 1.2b).

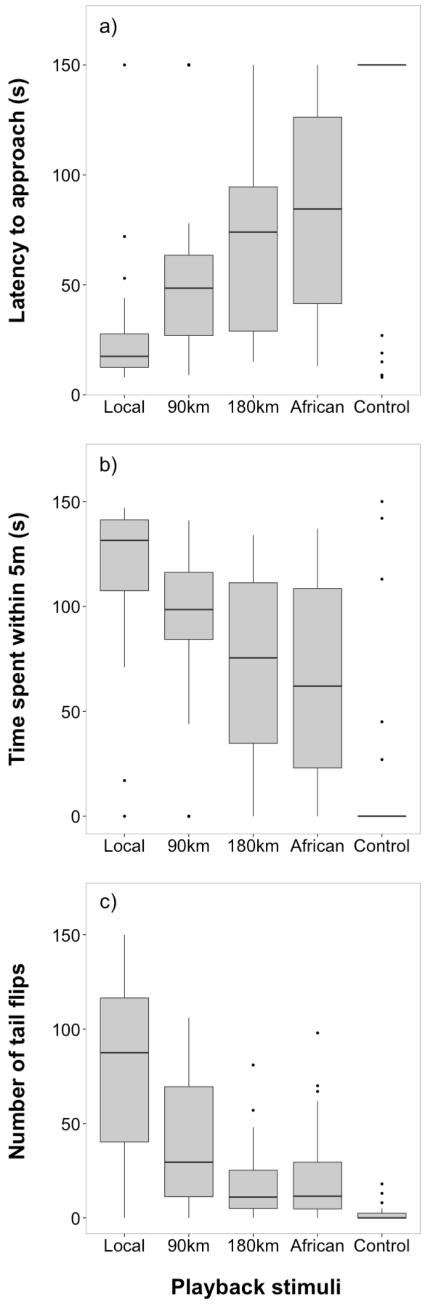

**Fig. 1.2 Playback experiment in males.** Behavioral response for (a) latency to approach, (b) time spent within 5 m, and (c) number of tail flips in response to European Stonechats from (1) the local population, (2) a population from 90 km distance, (3) a population from 180 km distance, (4) African stonechats, and (5) control stimuli (Winter wren). Males discriminated between local and foreign stimuli by responding more strongly to song of their own population. Box plots represent, from bottom to top: minimum, lower quartile, median, upper quartile and maximum. Dots indicate observations further than one s.d. away from the mean; n =28.

During the decoy experiment, the males' discrimination was less consistent than during the playback experiment ($z = -4.93$, $p <0,001$, Table 1.2c, Fig. 1.4). The latency to approach local decoy stimuli did not differ from other European ($z = -1.03$, $p =0.30$, Table 1.2c, Fig. 1.4) and African stimuli ($z = -1.52$, $p =0.13$, Table 1.2c, Fig. 1.4), but males approached the control decoy significantly later than all others ($z = -4.62$, $p <0.001$, Table 1.2c, Fig. 1.4). Breeding stage, trial order, date and time had no significant effect on the latency to approach (Table 1.2c).

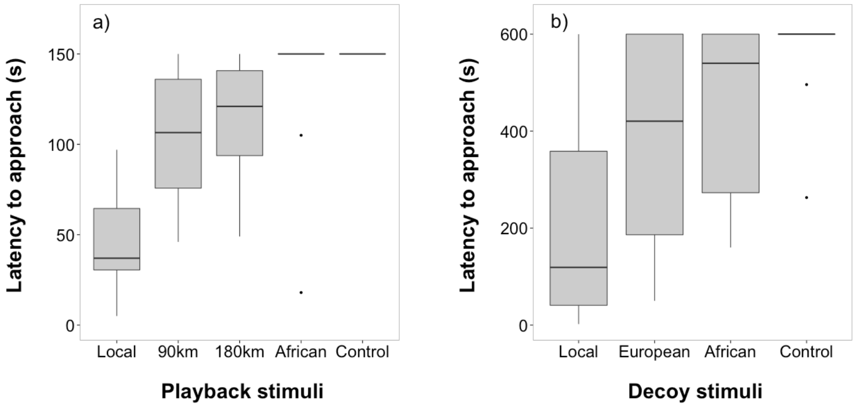

**Fig. 1.3 Playback and decoy experiment in females.** Behavioral response for the latency to approach within 5 m in (a) playback experiments in response to song of European Stonechats from (1) the local population, (2) a population from 90 km distance, (3) a population from 180 km distance, (4) African stonechats, and (5) control stimuli (Winter wren); and (b) decoy experiments in response to stimuli from (1) local, (2) European, (3) African, and (4) control stimuli (European Robin). Females distinguished between stimuli by approaching the local stimuli significantly faster than all other stimuli. Detailed description of boxplots as in Fig. 1.2; n =15.

Females showed finer discrimination (z =4.84, p <0.001, Table 1.2d, Fig. 1.3). They approached the local decoy with lower latency than decoys of populations from greater geographic distances (Table 1.2d, Fig. 1.3). Breeding stage, trial order, date and time showed no significant effect on the females' latency to approach the decoy (Table 1.2d).

There were no significant differences between different pairs, neither during the playback (z = −0.54, p =0.59, Additional file 1: Table S2a, Fig. 1.5) nor the decoy experiments (z = −0.39, p =0.70, Additional file 1: Table S2b, Fig. 1.5). Males approached the stimuli with significantly lower latency than females during both, the playback (z =4.78, p <0.001, Additional file 1: Table S2a, Fig. 1.5) and decoy experiment (z =5.88, p <0.001, Additional file 1: Fig. S2b, Fig. 1.5). Breeding stage, date and time did not influence the response patterns of pairs (Additional file 1: Table S2). Trial order had no influence on the behavioral response of pairs during the playback, and only a slight but significant effect during the decoy experiment (Additional file 1: Table S2). Birds tended to approach the stimulus with lower latency in the first two compared to later trials (Additional file 1: Fig. S4). A Spearman's correlation test was run to determine the relationship between the behavioral response of female and male mates within a pair. The latency to approach was correlated between females and males during the playback experiment ($r_s$ =0.51, p <0.001, n =15, Additional file 1: Table S3, Fig. S2), but not during the decoy experiment ($r_s$ =0.23, p <0.103, n =14, Additional file 1: Table S3, Fig. S2).

Further behaviors of males also differed in response to stimuli from different populations. Males remained significantly longer within 5 m of the stimulus of the local population than of all other populations during the playback (Table 1.3a, Fig. 1.2) and decoy experiment (Table 1.3b, Fig. 1.4). Males of the local population also discriminated between origin of the stimuli in the number of tail flips, an indicator of agitation. In response to playback, the number of tail flips differed significantly between stimulus origins (estimate =16.65, t = −10.58, CI = −19.82, 13.45, Table 1.4a, Fig. 1.2). The number of tail flips was highest when males were exposed to the local song and decreased with geographic distance of stimulus origin (Table 1.4a, Fig. 1.2). Breeding stage, trial order, date and time showed no significant effect on the number of tail flips (Table 1.4a). Similarly, males also differed significantly in their number of tail flips during the presentation of decoy stimuli (estimate = −18.43, t = −1.30, CI = −48.35, 11.16, Table 1.4b, Fig. 1.4). During trials of the local stimuli, males significantly flipped their tails more often than during all other trials (Table 1.4b, Fig. 1.4). Breeding stage, trial order, date and time showed no significant effect on the number of tail flips (Table 1.4b).

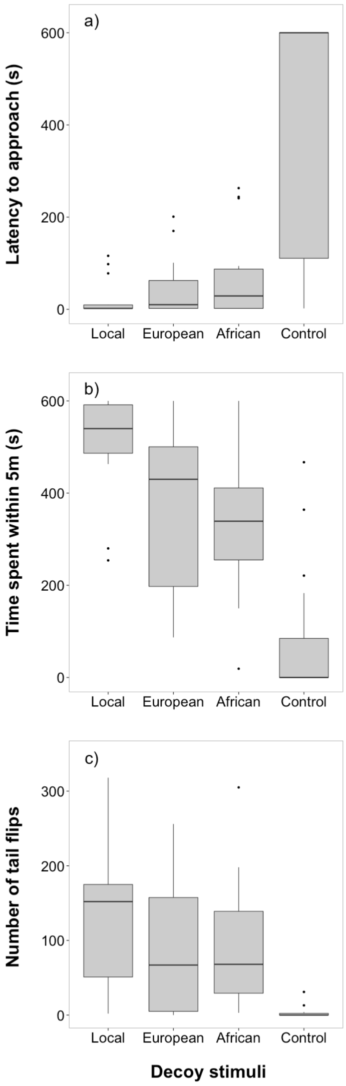

**Fig. 1.4 Decoy experiment in males.** Behavioral response for (a) latency to approach, (b) time spent within 5 m, and (c) number of tail flips to (1) local, (2) European, (3) African, and (4) control stimuli (European robin). Males discriminated between local and foreign stimuli by responding more strongly to decoys of their own population. Detailed description of boxplots as in Fig. 1.2; n =16.

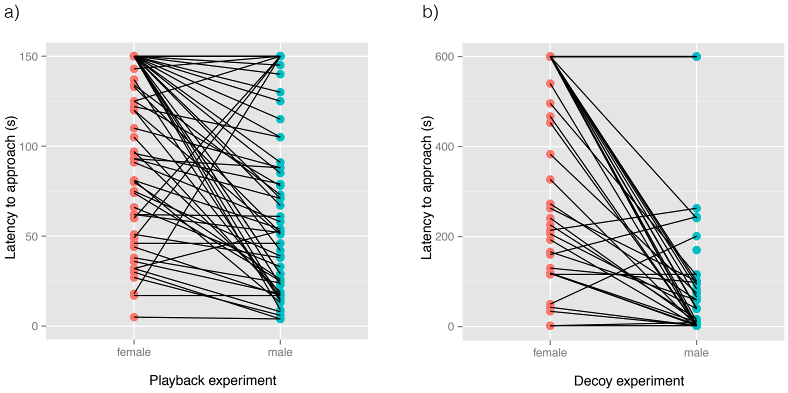

**Fig. 1.5 Response behaviors of female and male mates within pairs.** Shown is the latency to approach within 5 m for females (red) and males (blue) within pairs (connected dots) for (a) the playback (n =15 pairs) and (b) decoy experiments (n =14 pairs) for all stimuli trials. Females and males differ significantly in their response behavior, i.e. males approached the presented stimulus with lower latency.

**Table 1.3 Time spent within 5 m of the stimulus**

| | Fixed effects | Estimate | s.d. | CI 2.50% | CI 97.50% |
|---|---|---|---|---|---|
| (a) playback | local | 0.84 | 0.24 | 0.37 | 1.29 |
| | **90 km** | 0.18 | 0.21 | −0.22 | 0.62 |
| | **180 km** | −0.28 | 0.21 | −0.70 | 0.14 |
| | **African** | −0.37 | 0.22 | −0.80 | 0.05 |
| | **control** | −1.21 | 0.31 | −1.82 | −0.63 |
| (b) decoy | local | −1.93 | 0.45 | −2.75 | −0.97 |
| | **European** | −2.33 | 0.38 | −3.03 | −1.55 |
| | **African** | −1.97 | 0.36 | −2.65 | −1.24 |
| | **control** | 0.59 | 0.36 | −0.14 | 1.29 |

Results of generalized linear mixed model with estimates, standard deviation, and credible intervals using WinBUGS for (a) playback and (b) decoy experiment. Stimulus is defined as random factor to compare paths of all stimuli and thus correct for multiple testing. A behavioral response differs significantly from the local population if its credible intervals do not include the mean of the local population. Significant results are shown in bold.

**Table 1.4 Number of tail flips**

| | Fixed effects | Estimate | s.e.m | t | CI 2.50% | CI 97.50% |
|---|---|---|---|---|---|---|
| **(a) playback** | stimulus | −16.65 | 1.57 | −10.58 | −19.82 | −13.45 |
| | local | 21.34 | 25.35 | 0.84 | −30.85 | 73.32 |
| | **90 km** | −37.68 | 6.18 | −6.09 | −49.77 | −25.20 |
| | **180 km** | −57.62 | 6.16 | −9.35 | −70.01 | −45.05 |
| | **African** | −54.67 | 6.17 | −8.86 | −67.06 | −42.01 |
| | **control** | −74.16 | 6.46 | −11.48 | −87.36 | −60.74 |
| | breeding stage | 23.13 | 10.37 | 2.23 | 1.93 | 44.08 |
| | trial order | −3.10 | 1.44 | −2.16 | −5.97 | 0.44 |
| | date | 2.75 | 1.45 | 1.89 | −0.18 | 5.69 |
| | time | 0.49 | 0.50 | 0.97 | −0.54 | 1.51 |
| **(b) decoy** | stimulus | −18.43 | 14.13 | −1.30 | −48.35 | 11.16 |
| | local | 91.14 | 75.87 | 0.80 | −102.65 | 224.57 |
| | **European** | −10.80 | 43.36 | −0.25 | −106.25 | 83.18 |
| | **African** | −52.38 | 43.82 | −1.20 | −146.92 | 40.28 |
| | **control** | −48.50 | 44.02 | −1.10 | −144.07 | 46.80 |
| | breeding stage | −2.78 | 20.12 | −0.14 | −46.04 | 39.67 |
| | trial order | 59.11 | 16.39 | 3.61 | 24.44 | 93.31 |
| | date | −15.99 | 11.76 | −1.36 | −41.19 | 8.33 |
| | time | −0.28 | 1.00 | −0.28 | −2.40 | 1.82 |

Results of general linear mixed model with estimates, standard error, t-value, and credible intervals fitted by maximum likelihood. Estimates for the stimulus locations refer to differences from the intercept estimates, which represent the number of tail flips of the local population. Subjects were included as random intercepts to control for repeated measures. A behavioral response differs significantly from the local population if its credible intervals do not include the mean of the local population. Significant results are shown in bold.

# Discussion

This study reports clear differentiation in song traits of migratory European Stonechats over relatively short distances (90 km and 180 km from the focal group). By testing the behavioral responses to acoustic and morphological stimuli, we have also demonstrated the Stonechats' ability to discriminate between geographic origins of sexually selected traits in two modalities. The responses of both sexes during playback and decoy experiments were graded and declined with increasing geographic distance from the local population. The concordance of these responses and the significant preference for the closest population suggests potential for the evolution of reproductive isolation. Although at present we have confirmed this only for a single population.

Male and female Stonechats were similar in their behavioral discrimination, in contrast to results from other species. A recent study on Rufous-collared sparrows, *Zonotrichia capensis,* also reported discrimination between local and foreign stimuli, but the sexes differed in behavior (Danner et al. 2011). Females were presented with songs of the local, nearby nonlocal, and distant nonlocal dialect, and a control song from another bird species. They preferred the males' local song dialect to all other dialects tested, showing significantly more copulation solicitation displays. In contrast, males displayed only a low discrimination ability between dialects of geographically close populations (Danner et al. 2011). Similarly, in White-crowned sparrows, *Zonotrichia leucophrys*, females were more sensitive to geographic variation in song than males (Nelson and Soha 2004a). A study on hybridizing Pied flycatcher, *Ficedula hypoleuca*, and Collared flycatcher, *Ficedula albicollis*, revealed that females quickly recognize male species identity by song and have a strong preference for conspecific males resulting in assortative mating, and thus preventing further hybridization (Sætre et al. 1997; Haavie et al. 2004). In contrast, males of both species courted the heterospecific female and the conspecific female with similar intensity, thereby promoting hybridization. This lack of species recognition could be due to mating being less costly in males, which can inseminate several females over a short period, while females are constrained by the number of their eggs. Females, therefore, should not make mistakes in mate choice (Randler 2002). The fine discrimination ability of Stonechats indicates that females may mate assortative, and suggests that males may fight off particularly attractive sexual competitors with local dialects. We cannot disentangle male and female responses because we conducted simulated territorial intrusions in presence of both pair members. An influence of the mate is suggested by the correlation between mates during the playback experiment (Table S3, Fig. 1.5) and has been shown previously in Stonechats (Canoine and Gwinner 2005). Therefore, a crucial future step for a better understanding of the response to acoustic and morphological traits in Stonechats is to conduct experiments separately on females and males.

The local differentiation and consistent behavioral discrimination of song by origin of Stonechats, which migrate, was similar to that of resident species (e.g., indigobird *Vidua sp.* (Balakrishnan and

Sorenson 2006), Galapagos Sharp-beaked ground finch, *Geospiza difficilis* (Grant and Grant 2002), and song sparrow, *Melospiza melodia* (Patten et al. 2004)), but differed from findings in some migratory species. Among *Zonotrichia* sparrows, long distance migrants (e.g., *Z. l. gambelii)*, do not form song dialects (Nelson 1999), whereas in sedentary *Zonotrichia* subspecies (e.g., *Z. l. nutalli)* geographic song variation occurs (Baker 1982; Searcy et al. 2002). The lack of genetic diversification in migrants, in contrast to significant genetic structuring among dialect areas in non-migrants, supports the idea that migration may counteract population divergence and isolation (MacDougall-Shackleton and MacDougall-Shackleton 2001; Nelson and Soha 2004b; Soha et al. 2004).

Although the fine acoustic discrimination ability of Stonechats suggests potential behavioral barriers, its implications for geographic isolation are not fully clear, and partly depend on song plasticity, and ultimately on the mechanisms involved in song learning. In passerine birds, song is typically learned during a sensitive period early in life. In species like Stonechats that show geographic discrimination, males that subsequently disperse into ranges of other populations would face reduced mating prospects if an acoustic signature of the natal population remains in their repertoire (Helm et al. 2006). However, this could be offset if the males were able to learn new songs after the sensitive phase. For example, migratory nightingales were able to acquire new song types in their first singing season (Todt and Geberzahn 2003; Kiefer et al. 2006). In some species plastic song is based on an initial overproduction of learned songs during ontogeny (Kiefer et al. 2010). Such overproduction of learned songs has been suggested to be correlated with a migratory lifestyle (Marler and Peters 1982; Nelson et al. 1996). If present in Stonechats, plastic song learning could therefore counteract behavioral isolation. Furthermore, benefits of local song in sexual selection could be partly offset by the greater aversive response of local males. Dispersing females, in turn, may have no choice but to mate with a male singing a foreign dialect, which might also reduce population divergence. A modeling study by Ellers and Slabbekoorn (2002) also suggests that evolutionary implications of song dialects are not straightforward (Ellers and Slabbekoorn 2003). Although in the majority of scenarios genetic and vocal divergence were concordant, the type of song learning and intrasexual competition in males affected the evolutionary outcome. For Stonechats, to answer this question unambiguously would require population genetic analyses alongside analyses of song traits among populations (Mortega et al. 2015).

In our study, we found that Stonechats were also able to discriminate by morphological traits. Most studies of sexual selection do not explicitly test the role of simultaneous signaling with different sensory modalities, and instead focus on a single divergent signal or a suite of signals of the same modality (Uy and Borgia 2000; Irwin et al. 2001; Seddon and Tobias 2007). In contrast, explicitly testing for effects of multiple signals enables the detection of divergent signal use in discrimination (Baker and Baker 1990; Podos 2001). In Stonechats, we expected that culturally transmitted song may evolve more quickly, and thus could play a more important role for geographic clines than do morphological traits. We found that discrimination by song was more consistent than by morphological traits. The discrimination by song was sensitive to a geographic distance of only 90 km, whereas the decoy against which the birds visually discriminated originated from a population which breeds 1,000km away. A caveat in the interpretation of these differences are the different breeding stages during which the stimuli were tested: song stimuli were applied during egg-laying and incubation stages, when birds may be particularly responsive, whereas decoys were tested during nestling and fledgling stages. However, Stonechats are multi-brooded, and females may initiate additional clutches while males take care of fledglings, so that male intruders may well gain reproductive benefits at this time. Moreover, in a study on closely related African stonechats with similar experimental designs, but conducted during simultaneous breeding stages for playback and decoy experiments, the birds' discrimination by song was more consistent than that by morphology (unpublished data by KGM). Overall, our data cautiously suggest that song may be indeed the stronger discriminatory signal for Stonechats.

In the chestnut-bellied flycatcher, *Monarcha castaneiventris,* plumage color played a greater role than song for the intensity of aggressive response by territory-owners, although both signals mattered (Uy et al. 2009b). Chestnut-bellied flycatchers display more variation in plumage color than in song, which may indicate that plumage is more emphasized in sexual selection than song structure. The relative advantages of signaling with several modalities may be driven by the environment (Rowe and Guilford 1999). In general, acoustic signals can be transmitted over long distances and are ideal for long-range communication, whereas visual signals can be more limited and therefore more suitable for short-range communication (Laidre 2012). For Stonechats, which breed in open habitats, both signaling modes may be similarly important.

## Conclusions

Our study on Stonechats reveals geographic differentiation of sexually selected traits in a migratory songbird. Song traits differed significantly in populations of distinct geographic distances. Consistently, both sexes distinguished local morphological and especially acoustic phenotypes from those of foreign populations. These data demonstrate that variation in sexually selected traits of different modalities may contribute to geographic isolation over relatively short distances, and that the sexes may have similar sensitivity to incipient behavioral barriers. Maintenance or further divergence in these phenotypic traits could lead to assortative mating, reproductive isolation, and potentially speciation, in migratory Stonechats.

# Materials and Methods

## Subjects

Stonechats inhabit open habitats across a large extent of the Palearctic (Brewer 2003). The study population of European stonechats, *Saxicola torquata rubicola*, is located in northwest Germany (51°N, 6°30'E) and overwinters in the Mediterranean region, predominantly in north Africa (Flinks and Pfeifer 1987; Helm et al. 2006). The study population has been observed, measured and color-banded for individual recognition since 1976. Stonechats arrive at the breeding grounds early in spring (late February / March), establish a territory, and form seasonal pair bonds with two to three broods per season (Helm et al. 2006). After the postnuptial molt they start migrating towards the wintering grounds in early autumn (Schwabl et al. 2005; Flinks et al. 2008). In the present study, all focal pairs were ringed. We conducted regular checks twice per week to monitor the breeding activity and to define the breeding stage of each pair.

To test the discrimination ability of the local population, we collected songs from the local population (Düffel) and two nearby European Stonechat populations at distances of 90 km (Heubach) and 180 km (Wahner Heide) from the study area. Furthermore, we used songs and decoys of African stonechats from Kenya [distance 4,000 km, 61] and decoys from Stonechats from Austria [distance 1,000 km, 63]. Control species are explained below.

## Recording method and song analysis

Stonechats, in common with most passerines, spend a higher proportion of their time singing just before dawn than at other times of day (Krebs and Kacelnik 1983). During the onset of the breeding season, we recorded the dawn song of a minimum of 28 individuals from each European stonechat population (n = 3) for about ten minutes, using a Marantz PMD 661 solid state recorder (Osnabrück, Germany) and Sennheiser ME66/K6 directional microphones with windbreak (Georgsmarienhütte, Germany). To expand our set of stimuli, we also obtained 28 songs per species from African stonechats, *Saxicola torquata axillaris*, and Winter wrens, *Troglodytes hiemalis,* from the Macaulay Library (www.macaulaylibrary.org). These song recordings were conducted in the Rift Valley (Kenya) for African stonechats and New York State (United States) for Winter wrens.

We analyzed the songs of all five stimuli origins (sampling frequency: 44.1 kHz; resolution: 16 bit, Fig. 1.6) with the software Avisoft Sound Analysis Pro, version 5.1.09 (Raimund Specht, Berlin, Germany). We examined the song duration, number of elements per song, element rate (number of elements per second), minimum and maximum frequency, peak frequency (frequency of the highest amplitude sound), and bandwidth for all populations (Fig. S3). With the automatic parameter measurements setup, we obtained the minimum and maximum frequency values measuring at a standard decibel threshold (here -20 dB, total option) below the peak in the power spectra (Zollinger et al. 2012).

To assess the song repertoire size, we analyzed 100 consecutive songs of each male Stonechat (n = 20) from the local population. In Stonechats, a song typically consists of a sequence of motifs, and these in turn each contain several consecutive elements (Fig. S3). They are stereotypically repeated at a constant rate, and thereby distinguishable from all other song types. In general, song motifs, rather than complete songs, are shared within a population. The mean song repertoire consists of 16 ± 3.06 unique song types.

## Playback experiment

To reveal behavioral responsiveness of male and female Stonechats to songs of different dialects, we performed a field-based playback experiment by simulating a territorial intrusion with songs of distinct dialects during the egg laying or incubation stage (13th – 26th April, 2011). Each subject received five playback trials with the sequence of exposure determined by a randomized block design created by Randlist 1.2 (DatInf GmbH, Tübingen, Germany) to account for bias by trial order effects. Stimuli strings consisted of songs from the three European and the single African Stonechat populations. As a control, we used song of heterospecific Winter wrens, *Troglodytes hiemalis*. This species was chosen following the rationale by Grant and Grant (Grant and Grant 2002), using a species that is similar in note structure and frequency range, but has never been heard by the tested birds.

To avoid inclusion of rare motifs, we selected song types with defined common motifs, which are shared between members of a population, and thus are representative of each population. However, this implies that the interpretation of song discrimination between populations should be taken with caution. The standardized stimuli selection of common and locally shared songs most likely excluded overlapping songs among populations. Their incorporation may have led to a slight decrease in the discrimination ability, and thus a potential overestimation of the responsiveness. To increase the number of independent samples, and thus improve the reliability and external validity, we generated a unique stimulus for each trial (Kroodsma 1989). Each stimulus song was only used once for the entire study. We tested females and males simultaneously on their territories, and therefore each stimulus string was used once for both sexes (i.e. 28 x 5 = 140 unique playback stimuli). Each stimulus comprised song types from one individual's recording following the natural syntax of Stonechat song. We used 25 unique songs in total for each stimulus string, which were filtered (1 kHz high-pass filter) and normalized in peak amplitude (i.e. the amplitude of each song was adjusted to 75 % of the maximum amplitude). Songs were divided by pauses of 4.5 seconds. A trial comprised all five population stimuli played back consecutively in a random order, each with a duration of 150 seconds. Each stimulus string was followed by at least 150 seconds of silence. To ascertain a comparable behavioral response of the latency to approach for each stimulus, playback strings only started when the focal bird (males = 28, females = 15) was at a distance of at least 10 m from the caller (longest silence between consecutive strings 285 s). Hence, each trial was performed on an individual bird for a period of about 25 minutes in total depending on the start times of the consecutive playback stimuli. Stimuli were broadcasted with the caller Foxpro Scorpion X1B (digital game caller, FOXPRO Inc. Lewistown, USA), which could be operated with a remote control. It was mounted on top of a bush in the central area of a territory such that it was widely audible. Response songs were recorded during the entire trial. However, acoustic responses to the playback were rare, and thus were not included in further analysis.

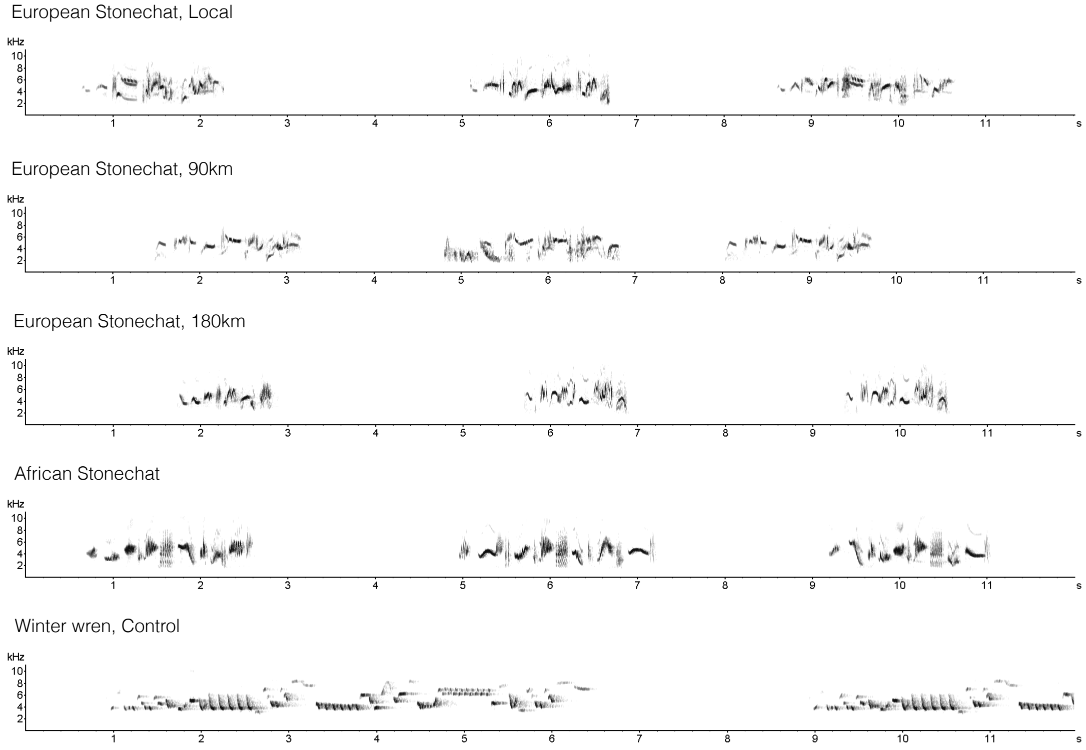

**Fig. 1.6 Exemplary frequency spectrograms of acoustic stimuli used for the playback experiment**. Stimuli strings of European stonechat consisted of songs from members of (a) the local population and from populations at distances of (b) 90 km, and (c) 180 km from the study area. Furthermore, we played back song of (d) African stonechats, and (e) Winter wrens as a control.

## Decoy experiment

We conducted a second experiment to test the responsiveness towards morphological traits by using a stuffed decoy simulating a territorial intrusion. During the nestling or fledgling stage we placed the decoy (male in full adult plumage protected by an inconspicuous cage) in the center of respective territories for ten minutes in total for each trial. Decoy stimuli consisted of males from (a) local Stonechats, (b) European Stonechats from Austria [distance 1,000 km, 63], (c) African Stonechats from Kenya [distance 4,000 km, 61], and as a control (d) European Robins, *Erithacus rubecula*. From extensive observations, we know that Stonechats aggressively chase off other small insectivorous passerines with similar feeding habits. European robins meet this criterion but their preference of deciduous wooded habitats limits their familiarity to Stonechats. To avoid pseudo-replication we randomly chose from five different decoys per stimulus for each trial. Each focal Stonechat (male = 16, female = 14) received all stimuli in a randomized and balanced order. We conducted each trial on a different date (5th-18th May, 2011) during morning hours with two days pause between trials.

## Behavioral measurements

All behavioral responses were observed from a distance of about 30 m and were documented continuously by dictating to the Marantz PMD 661. To quantify behavior, we used descriptors that are commonly used to measure responses to territorial intrusions and mate attraction (McGregor 1992) including studies in Stonechats (Canoine and Gwinner 2002). Specifically, we measured the latency of a bird to approach the playback or decoy within 5 m; the time a bird spent within this 5 m zone; and the number of tail flips, which are defined as up- and downward movements of the entire tail and indicate agitation in Stonechats (Brewer 2003). The descriptive statistics of all behavioral responses can be found in the supplements (Table S4).

## Statistical analysis

All statistical analyses were performed with the software R v. 3.1.0 (R Core Team 2014). Tests were two-tailed and significance was accepted at $\alpha$=0.05. We used principal component analyses (PCA, R package *FactoMineR* (Husson et al. 2014)) to compare song traits between groups (with and without both control groups) and then tested the first principal component in a general linear model (LM, R package *lme4* (Bates et al. 2012)) to identify the relationship between song traits and geographic distance of song origins from the local population. The latencies to approach within 5 m to the different stimuli were analyzed using mixed-effects cox models (survival model) fitted by maximum likelihood accounting for breeding stage, randomized trial order, date and time (coxme, R package *survival* (Therneau 2011)). Subjects were included as random intercepts to control for repeated measures. A Spearman's correlation was run to determine the relationship of the behavioral response between paired females and males. For the time spent within 5 m we used a generalized linear mixed model with a beta distribution and stimulus as random factor using WinBUGS software 1.4 (GLMM, R package *R2WinBUGS* (Lunn et al. 2000; Sturtz et al. 2005)). In WinBUGS we focused exclusively on differences between stimuli. We defined stimulus as a random factor to compare paths of all stimuli, and thus correct for multiple testing. The response number of tail flips in males was analyzed with a general linear mixed model fitted by maximum likelihood methods (LMMs, R package lme4 (Bates et al. 2012)) controlling for breeding stage, trial order, date and time. Subjects were included as random intercepts to control for repeated measures. Predictions from the general and generalized linear mixed models (Bayesian methods) were calculated as the median of their posterior distributions, and the 2.5 and 97.5% credible intervals (CI).

## Acknowledgements

All procedures follow NIH guidelines for the Care and Use of Experimental Animals and were conducted under the permission of the responsible authorities (West Münsterland, Kreis Borken, North-Rhine-Westphalia, Germany). The authors thank Martin Wikelski for providing equipment. We also thank Davide M. Dominoni, Fränzi Korner-Nievergelt, Beate Apfelbeck, Wolfgang Goymann, Manfred Gahr, Michaela Hau, Jim Caryl and three anonymous reviewers for providing valuable discussion and feedback on the manuscript. KGM is a member of the International Max Planck Research School for Organismal Biology and is funded through the German Research Foundation (DFG grant HE3488/5-1).

## Additional file

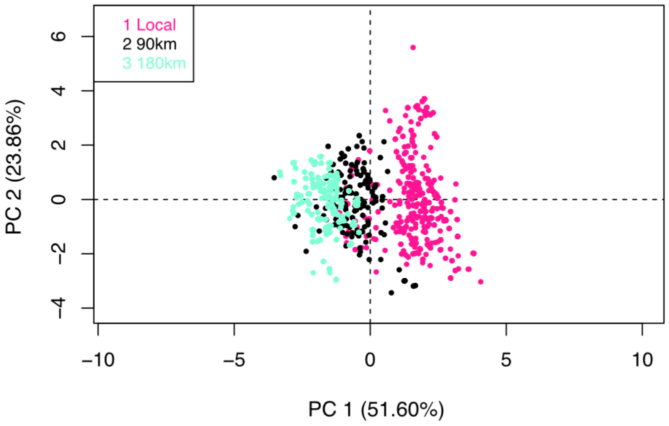

**Fig. S 1 Geographic variation in the song of European stonechats as quantified by principal component analysis.** Shown is (a) the variation in song structure of European Stonechats from (1) the local population, (2) a population from 90 km distance, and (3) from 180 km distance based on a principal component analysis (for details, see Table S1). Based on the first principal component, the song of the focal population differed significantly from the two neighbouring populations (90 and 180 km). The two latter populations did not differ significantly in their song from each other. However, differences in song traits reflected the geographic distances to the local population

**Table S 1 Song traits of three European Stonechat populations**

| | PC 1 | PC 2 | PC 3 | |
|---|---|---|---|---|
| (a) | | | | |
| song duration | 0.31 | 0.53 | 0.05 | |
| no. of elements | -0.15 | 0.74 | -0.06 | |
| element rate | 0.39 | -0.39 | 0.15 | |
| peak frequency | -0.02 | 0.10 | 0.96 | |
| min. frequency | 0.49 | 0.01 | 0.09 | |
| max. frequency | -0.48 | -0.13 | 0.19 | |
| bandwidth | -0.51 | -0.06 | 0.03 | |
| | | | | |
| eigenvalue | 3.61 | 1.67 | 1.04 | |
| % variance | 51.60 | 23.86 | 14.89 | |
| | | | | |
| (b) | | | | |
| **fixed effects** | **estimate** | **s.e.m** | **t** | **p** |
| | | | | |
| **intercept** | -0.04 | 0.11 | -0.35 | 0.73 |
| **90 km** | -1.02 | 0.15 | **15.51** | **<0.001** |
| **180 km** | 2.29 | 0.15 | **-6.67** | **<0.001** |

(a) Factor loadings of the principal component analysis for seven song traits of (1) the local population, (2) the population from 90 km distance, and (3) the population from 180 km distance. (b) Results of general linear model testing whether the first principal component (PC1) differed between songs from different locations, estimated by maximum likelihood methods. Estimates for the different song locations refer to differences from the intercept estimate, which represents song traits of the local population. Subjects were included as random intercepts to control for repeated measures. 'Significant' differences are shown in bold; n = 81.

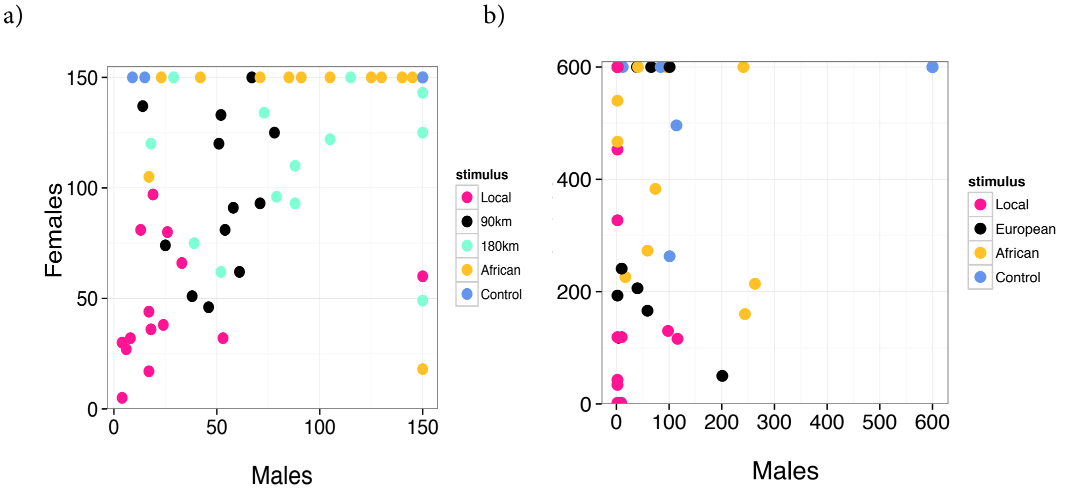

**Fig. S 2 Female and male behavioural responses.** The figures show the latency to approach within 5 m of a stimulus in females and males during (a) playback experiments in response to song of European Stonechats from (1) the local population, (2) the population from 90 km distance, (3) the population from 180 km distance, (4) African stonechats, and (5) control stimuli (Winter wren); and (b) decoy experiments in response to stimuli from (1) local, (2) European, (3) African, and (4) control stimuli (European Robin).

**Table S 2 Behavioural response between pairs**

| | estimate | hazard ratio | s.e.m. | z | p |
|---|---|---|---|---|---|
| **(a) playback** | | | | | |
| pairs | -0.07 | 0.93 | 0.14 | -0.54 | 0.59 |
| **sex (female vs. male)** | 1.19 | 3.27 | 0.25 | 4.78 | **<0.001** |
| breeding stage | -0.36 | 0.70 | 0.76 | -0.48 | 0.63 |
| trial order | 0.07 | 1.07 | 0.16 | 0.41 | 0.68 |
| date | 0.06 | 1.06 | 0.16 | 0.36 | 0.72 |
| time | -0.009 | 0.99 | 0.10 | -0.01 | 0.99 |
| **(b) decoy** | | | | | |
| pairs | -0.02 | 0.98 | 0.04 | -0.39 | 0.70 |
| **sex (female vs. male)** | 1.71 | 5.52 | 0.29 | 5.88 | **<0.001** |
| breeding stage | 0.03 | 1.03 | 0.19 | 0.15 | 0.88 |
| **trial order** | -0.36 | 0.70 | 0.17 | -2.08 | **0.04** |
| date | 0.09 | 1.10 | 0.12 | 0.79 | 0.43 |
| time | -0.01 | 0.99 | 0.01 | -1.12 | 0.26 |

Results of the cox mixed-effects model with estimates, hazard ratio, standard error, z-value, and p-value fitted by maximum likelihood of the latency to approach controlling for sex, breeding stage, trial order, date and time. Subjects were included as random intercepts to control for repeated measures. 'Significant' differences are shown in bold.

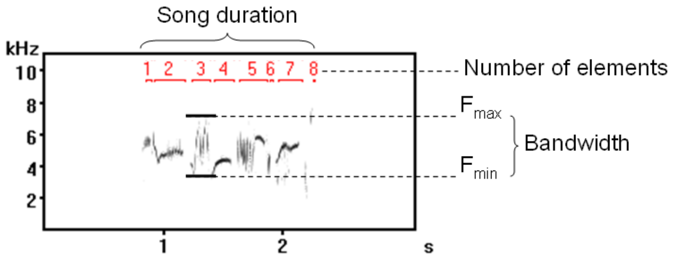

**Fig. S 3 Spectrogram of an exemplary song in European stonechats.** Indicated are typical measured song traits of Stonechats, i.e. song duration, total number of elements, minimum and maximum frequency, and bandwidth.

**Table S 3 Correlation of behavioural responses between females and males during playback and decoy experiment**

| | rho | p | n |
|---|---|---|---|
| **playback** | 0.51 | <0.001 | 15 |
| **decoy** | 0.23 | 0.103 | 14 |

Results of Spearman's rank correlation test for the latency to approach within 5 m of the stimulus between pair mates; data shows correlation coefficient rho, p-value, and sample size.

**Table S 4 Descriptive statistics of behavioural responses for a) playback (n = 28) and b) decoy (n = 16) experiment.**

| stimulus | time spent within 5m (s) | number of tail flips | latency to approach (s) | latency to approach in females (s) |
|---|---|---|---|---|
| **a) playback** | | | | |
| **local** | 117.36 ± 36.88 | 75.11 ± 47.38 | 22.21 ± 29.77 | 46.07 ± 26.71 |
| **90 km** | 92.07 ± 40.04 | 38.54 ± 32.10 | 50.54 ± 41.19 | 104.50 ± 37.85 |
| **180 km** | 70.79 ± 44.90 | 17.82 ± 19.88 | 73.71 ± 46.15 | 112.79 ± 33.47 |
| **African** | 63.54 ± 47.08 | 21.21 ± 25.06 | 83.50 ± 8.19 | 137.36 ± 36.39 |
| **control** | 19.88 ± 46.02 | 2.38 ± 4.60 | 115.83 ± 60.59 | 150.00 ± 0 |
| **b) decoy** | | | | |
| **local** | 307.21 ± 223.89 | 121.36 ± 168.80 | 23.50 ± 40.79 | 212.80 ± 224.43 |
| **European** | 272.50 ± 229.62 | 111.93 ± 135.40 | 50.00 ± 65.40 | 381.17 ± 233.22 |
| **African** | 389.27 ±194.67 | 93.73 ± 109.52 | 69.80 ± 97.65 | 451.00 ± 175.40 |
| **control** | 314.67 ± 218.86 | 92.60 ± 134.66 | 460.70 ± 239.98 | 566.08 ± 95.49 |

Shown are the mean ± standard deviation of the time spent within 5 m, the number of tail flips and the latency to approach of males ($n_{playback}$ = 28, $n_{decoy}$=16) and females ($n_{playback}$ = 15, $n_{decoy}$=14) for (1) the local, (2) 90 km, (3) 180 km, (4) African, and (5) control stimuli.

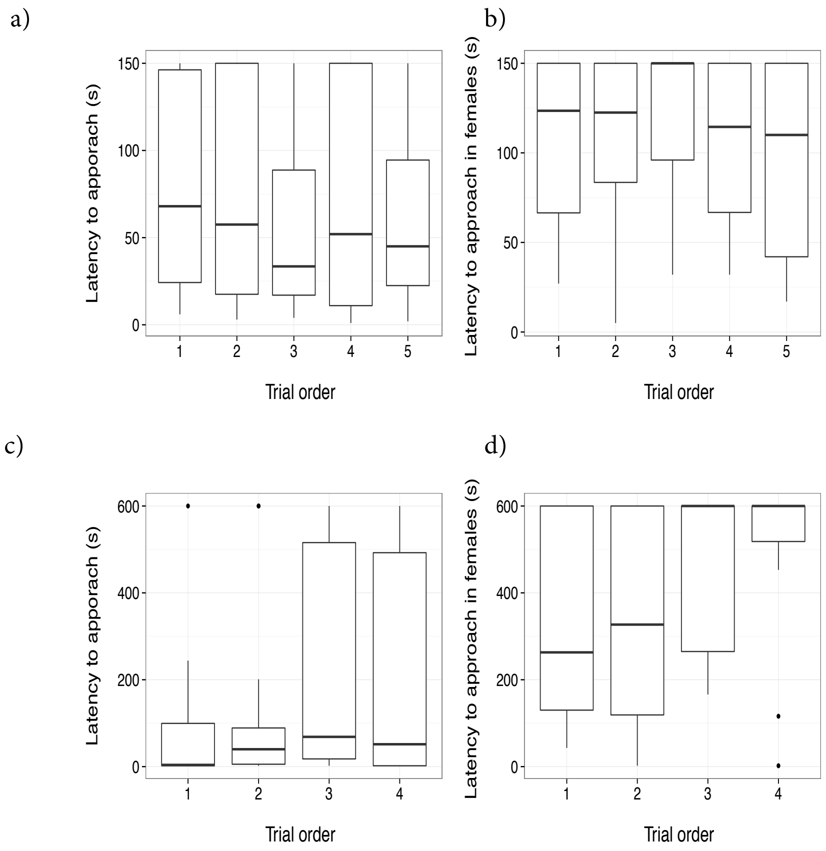

**Fig. S 4 Trial order for the latency to approach during the playback experiment** in (a) males and (b) females, and during the decoy experiment in (c) males and (d) females. Box plots represent, from bottom to top: minimum, lower quartile, median, upper quartile and maximum. Dots indicate observations further than one s.d. away from the mean.

# PART 2: Seasonal endocrine regulation of song and behavior

# *CHAPTER 2* Associated and disassociated patterns in hormones, song, behavior and brain receptor expression between life-cycle stages in male Black redstarts, *Phoenicurus ochruros*

*Apfelbeck B, Mortega KG, Kiefer S, Kipper S, Michiel Vellema M, Villavicencio CP, Gahr M, Goymann W. 2013 General and Comparative Endocrinology 184 93–102*

# Abstract

Testosterone has been suggested to be involved in the regulation of male territorial behavior. For example, seasonal peaks in testosterone typically coincide with periods of intense competition between males for territories and mating partners. However, some species also express territorial behavior outside a breeding context when testosterone levels are low and, thus, the degree to which testosterone facilitates territorial behavior in these species is not well understood. We studied territorial behavior and its neuroendocrine correlates in male black redstarts. Black redstarts defend territories in spring during the breeding period, but also in the fall outside a reproductive context when testosterone levels are low. In the present study we assessed if song output and structure remain stable across life-cycle stages. Furthermore, we assessed if brain anatomy may give insight into the role of testosterone in the regulation of territorial behavior in black redstarts. We found that males sang spontaneously at a high rate during the nonbreeding period when testosterone levels were low; however the trill-like components of spontaneously produced song contained less repetitive elements during nonbreeding than during breeding. This higher number of repetitive elements in trills did not, however, correlate with a larger song control nucleus HVC during breeding. However, males expressed more aromatase mRNA in the preoptic area – a brain nucleus important for sexual and aggressive behavior – during breeding than during nonbreeding. In combination with our previous studies on black redstarts our results suggest that territorial behavior in this species only partly depends on sex steroids: spontaneous song output, seasonal variation in trills and non-vocal territorial behavior in response to a simulated territorial intruder seem to be independent of sex steroids. However, context-dependent song during breeding may be facilitated by testosterone – potentially by conversion of testosterone to estradiol in the preoptic area.

# Introduction

Most animals live in highly seasonal environments that vary, for example, in temperature and precipitation and consequently in the availability of resources such as food. To survive and maximize their reproductive success animals need to cope with these largely predictable changes and time their life cycle accordingly. Hormones play a major role in the development and transition between life-cycle stages (Jacobs and Wingfield 2000; Wingfield et al. 2001) and testosterone has been identified as an important player in regulating the breeding life-cycle stage of male vertebrates: it is required for spermatogenesis, the development of secondary sex characteristics and it facilitates sexual and territorial behaviors (Nelson 2005). Accordingly, testosterone levels are often highest at the beginning of the breeding season when interactions among males, song and sexual activities are most intense (Dawson 1983; Silverin et al. 1986; Ball and Wingfield 1987; Morton et al. 1990; Wingfield et al. 1990; Silverin 1993; Van Duyse et al. 2003). However, the correlation between behaviors expressed in a territorial context and testosterone levels is not always that straight-forward: many temperate-zone songbird species, for example, defend territories and sing outside the breeding season when testosterone levels are low (Schwabl 1992; Wingfield and Hahn 1994; Canoine and Gwinner 2002; Landys et al. 2010; Apfelbeck and Goymann 2011). Testosterone could still facilitate territoriality in these species: testosterone precursors may be derived from non-gonadal sources and metabolized to testosterone directly in the brain (Soma et al. 2000a; Soma and Wingfield 2001) or the brain may have a higher sensitivity for low levels of the hormone (Fusani et al. 2007). In some species, however, testosterone facilitates territorial behavior during the breeding period, but does not appear to facilitate territorial behavior during the nonbreeding season (Schwabl and Kriner 1991; Hau et al. 2000; Canoine and Gwinner 2002; Landys et al. 2010; Hau and Beebe 2011; Marasco et al. 2011). In rufous-collared sparrows (*Zonotrichia capensis*) territorial behavior seems to be independent of testosterone even during breeding (Ignacio T et al. 2004; Moore et al. 2004). In other species, that defend territories and sing both in- and outside the breeding season, these behaviors seem to differ in these two contexts (Moore 1988). For example, during breeding song may contain more repetitive elements (Dewolfe et al. 1974; Smith et al. 1997; Leitner et al. 2001; Voigt and Leitner 2008), longer songs (Riters et al. 2000) or song may be more stereotyped (Smith et al. 1997) than during nonbreeding. In the latter cases testosterone during breeding may activate these changes in territorial behaviors and song. In song sparrows (*Melospiza melodia*), for example, seasonal changes in song have been correlated with the size of the HVC, a brain nucleus of the song control system in songbirds (Nottebohm et al. 1976) that is considered to control motor output during singing (Yu and Margoliash 1996; Brenowitz 1997). The HVC is sensitive to androgens (Gahr and Metzdorf 1997) and its size is thought to depend at least partly on circulating testosterone levels (Nottebohm 1980; Sartor et al. 2005). Furthermore, the sensitivity to testosterone may change within the HVC depending on season (Gahr and Metzdorf 1997; Soma et al. 1997). It has been argued that a larger HVC during breeding is related to a larger song repertoire, a higher song rate and facilitation of a more complex song in song sparrows (Brenowitz 1997; Smith et al. 1997), but see (Gahr 1997). In other species such as canaries (*Serinus canaries*) and black-capped chickadees (*Poecile atricapillus*), however, seasonal changes in song are not related to HVC size (Fusani et al. 2000; Smulders et al. 2006). Furthermore, testosterone may influence the motivation to sing and the song rate in a reproductive context by activating song areas outside the song control system, e.g. by aromatization of testosterone to estrogens in the preoptic area (Foidart et al. 1998; Riters et al. 2000). The preoptic area has been shown to play an important role in the regulation of estrogen-dependent aggressive behavior (Schlinger and Callard 1990; Silverin et al. 2004).

Hence, although it is well accepted that testosterone plays a role in the organization and activation of song (Bolhuis and Gahr 2006) and territorial behavior (Wingfield et al. 2006) in the breeding season, it is still unclear to what extent testosterone facilitates these behaviors in species that sing and defend territories outside the breeding season.

The black redstart (*Phoenicurus ochruros*) is a temperate-zone songbird species that defends a territory and sings during the breeding season in spring and also during nonbreeding in fall. Black redstarts are socially monogamous and both females and males provide parental care (Landmann 1996; Wang et al. 2005). Males defend a territory and sing during the breeding season in spring and early summer. During late summer (mid-August‑mid-September) they molt and show a decrease in singing activity. Afterwards they express a pronounced period of fall territoriality and song activity until the end of October just before they start migration (Weggler 2000).

To investigate whether testosterone may facilitate song and territorial behavior in different life-cycle stages, we compared testosterone (obtained via blood samples), territorial behavior, spontaneous song, and the distribution of hormone receptors in the brain of male black redstarts during breeding and nonbreeding territoriality. We focused on brain areas relevant for singing (forebrain song control nuclei) and aggressive behavior (diencephalon). We describe the expression pattern of androgen receptor-, estrogen receptor- and aromatase mRNA of male black redstarts during breeding and nonbreeding territoriality and determine whether HVC size and aromatase expression in the preoptic area differ between life-cycle stages and correlate with testosterone levels, territorial behavior and spontaneous song. If song output and structure differ with life-cycle stage, we expected males to have a larger HVC during breeding than outside the breeding season (Smith et al. 1997). As aromatase expression in the preoptic area has been shown to play an important role in the expression of reproductive behaviors (Balthazart et al. 2010), we expected a higher expression of aromatase mRNA in that area during breeding compared to the nonbreeding fall territorial phase.

We compared song output and song structure between breeding and nonbreeding territoriality to test if they differ between life-cycle stages. If testosterone activates song during the breeding season we expected that males produce more spontaneous song during breeding in spring than during nonbreeding in fall. Similar to other species, parts of the song of black redstarts contain repetitive elements and males increase the number of these elements in an agonistic context (Apfelbeck et al. 2012). If testosterone changes the structure of song during breeding, we expected to find significant differences in the structure of spontaneous song between the breeding and the nonbreeding season.

# Material and Methods

## Study period and study site

Free-living male black redstarts were challenged with simulated territorial intrusions and caught in 2008 (April 1-June 12; September 19-October 6), 2009 (July 3-August 13) and, 2010 (June 28-July 31) in Upper Bavaria, Germany (N 47°, E 11°, 500-600 m above sea level). These males contributed to different experiments (see below); however most of them were bled and contributed to the seasonal testosterone profile presented. Furthermore, some of the hormone data from the early breeding season and the fall nonbreeding period as well as the behavioral responses to simulated territorial intrusions were collected as part of a different study and have been previously published (Apfelbeck et al. 2011). The comparison between the two territorial phases (during breeding in spring and nonbreeding in fall) was conducted in a cross-sectional manner (studying different birds in each phase). Breeding stages for the seasonal hormone profile were determined by behavioral observations of males and females during capture. Before capture, we mapped territories by determining boundaries through frequently used singing posts. After capture, territorial males were followed throughout the breeding season and the presence of focal males in the respective territories was confirmed through observation or the use of playback (i.e. we played back black redstart song until the territory owner responded by approaching the loud speaker or by singing or for at least 10 min). The main focus of this study was a comparison of hormone levels, behavior, song and brain between early breeding season territory establishment and fall nonbreeding territoriality. In addition, hormonal data were obtained in more detail throughout the whole breeding season and during molt. Therefore, for the hormone part of the manuscript, the breeding period was further divided into several substages (incubation, nestlings and fledglings of first brood, nestlings and fledglings of second brood).

## Simulated territorial intrusion experiments

Territorial behavior was assessed by simulated territorial intrusion experiments (STI). For a full description of the territorial intrusion experiments see (Apfelbeck and Goymann, 2011). Briefly, a stuffed decoy (in full adult plumage, three specimens) along with black redstart song was placed into the territory and the behavioral responses of the territory owner were recorded from a distance. We used five different playback strings with species-typical song in random order (wav.files, each repeated at a rate of eight strophes per min) that were played back from a loudspeaker at a sound pressure levels of 65 dB SPL at 1 m (as measured with a CEL 573.B1 Sound Level Analyser) directly below the decoy. We recorded the following behaviors of the territory owner during 10 min of vocal playback: (1) latency to respond to the STI either by singing or approaching the decoy, (2) the first time the male was in a 5 m radius around the decoy, (3) the total time the male spent in this 5 m radius, (4) the total time the territory owner fluffed its feathers, and (5) the number of head nods. Furthermore, we noted whether the male attacked the decoy or sang at any time during the STI.

## Capture and blood sampling

Males were caught after the STI experiments or while searching for food to obtain blood for hormone analysis (see also Apfelbeck and Goymann, 2011 and Apfelbeck et al., 2011). They were caught after STI experiments to obtain brains for mRNA expression analysis. Birds were caught in mealworm-baited ground traps or tree traps (in the case of simulated territorial intrusion experiments, some traps were attached close to the decoy and were opened after ten minutes and the playback continued until capture of the territory holder). Immediately upon capture (178 ± 112 s), a blood sample (~120 $\mu$l) was taken from the wing vein and collected into heparinized capillaries. We checked if males were molting body, wing, and/or tail feathers and banded them with a numbered aluminum ring (Vogelwarte Radolfzell) and a combination of two color rings. All experimental procedures were approved by the governmental authorities of Upper Bavaria.

## Recording and analysis of spontaneous song

Adult (≥2 years) territorial male black redstarts were recorded with a Sennheiser directional microphone (ME66/K6) connected to a Marantz solid state recorder PMD 660 (sampling frequency: 44.1 kHz; resolution: 16 bit) during breeding territorial establishment in spring 2009 (April 9‑27, n = 20) and a different set of individuals during the nonbreeding territorial phase in fall 2009 (September 22‑October 7, n = 12). Songs were analyzed using Avisoft-SASLab Pro software, version 4.51. Recordings were visualized in spectrograms (settings: sample rate 22,050 Hz, FFT = 256 points, Hamming-Window, Overlap: 50%). We determined the number of songs by visual inspection and selected songs of sufficient quality (low background noise) for further sound analysis. Each song of black redstarts can be divided into three distinct parts (part A, B, and C, e.g. (Cucco and Malacarne, 1999) with a pause of varying length between part A and B. We measured the duration of part A, B, C, the total song and the duration of pauses between A and B. We counted the number of elements of part A and C (mean of max. 20 songs). We also determined the frequency bandwidth and the maximum frequency of part A, B, and C using the automatic parameter measurement function (threshold −20 dB) in Avisoft (mean of max. 10 renditions of high-quality songs).

## Plasma separation and hormone analysis

Plasma was immediately separated by centrifugation with a Compur Minicentrifuge (Bayer Diagnostics). The amount of plasma was measured with a Hamilton syringe and stored in 500 μl ethanol (Goymann et al. 2007b). After returning from the field samples were stored at −80 ° C. Testosterone concentration was determined by direct radioimmunoassay (RIA, following (Goymann et al. 2007b; Apfelbeck et al. 2011). Samples were measured in duplicates in four assays. Mean ± SD efficiency of the extraction with dichloromethane was 92.5 ± 0.1%. The lower limits of detection of the assays were determined as the first values outside the 95% confidence intervals for the zero standard ($B_{max}$) and were 6.7, 6.6, 6.0, and 8.0 pg/tube respectively. The intra-assay coefficients of variation were 4.7%, 4.2%, 3.1%, and 7.9%, respectively. The inter-assay variation was 6.4 ± 1.8%. As the testosterone antibody shows significant cross-reactions with 5α-dihydrotestosterone (44%) our measurements may include a fraction of this potent androgen.

## Tissue collection

Brains and testes were collected between April 14th‑28th in 2008 (n = 8) and between September 2th 1‑24th in 2009 (n = 8). Upon capture, birds were immediately killed by decapitation and brains removed from the skull, frozen on dry ice and stored at -80 ° C until further analysis. We also recorded the length and width of the left and right testis using calipers and calculated testis volume as the volume of an oval body $(4/3 * \pi * (\text{width}/2)^2 * (\text{length}/2))$. Frozen brains were cut into 20 μm sagittal sections on a cryostat microtome (Leica Microsystems GmbH, Wetzlar, Germany) and collected on Superfrost object slides (Menzel GmbH, Braunschweig, Germany) in five parallel series. One series of brain sections was selected for Nissl staining and used to provide anatomical landmarks for later interpretation of in situ hybridization results. The remaining series were used for in situ hybridization of adjacent sections for androgen receptor, estrogen receptor α and aromatase.

## In-situ hybridization

Riboprobes were synthesized from cDNA previously cloned from zebra finch androgen and estrogen receptor α mRNA (Gahr and Metzdorf 1997) and canary aromatase mRNA (Metzdorf et al. 1999). Antisense and sense $^{35}$S-CTP-labeled probes were transcribed from the T7 and SP6 promoter region of a pGEM7Zf + vector using the Riboprobe System (Promega, Madison, WI). Brain sections were fixed in a 4% formaldehyde solution in phosphate-buffered saline (PBS; 0.01 M; pH 7.4) for 5 min, washed in DEPC-treated PBS, and incubated in 0.25% acetic anhydride in ethanolamine (TEA; 0.1 M; pH 8.0) for 10 min to reduce non-specific binding. After a washing step in 2× standard saline citrate (SSC), sections were dehydrated in serially increasing percentages of ethanol, and left to dry at room temperature. Sections were hybridized under a cover slide with $^{35}$S-CTP-labeled sense or antisense riboprobes ($0.4 \times 10^6$ cpm/slide) in hybridization buffer with 50% formamide and 10% dextran sulfate overnight at 55 ° C. After hybridization, slides were immersed in 2× SSC at room temperature to remove the cover slides and incubated in RNase A (20 ug/ml) for 30 min at room temperature. Sections were then consecutively washed for 30 min in 2× SSC at 50 ° C, 0.2× SSC at 55 ° C, and 0.2× SSC at 60 ° C, dehydrated in ethanol containing 0.3 M ammonium acetate, and dried for 1 h at room temperature. Finally, slides were exposed to Kodak BioMax MR film (Sigma-Aldrich Co., St. Louis, MO) in lightproof boxes for 3 weeks at room temperature, developed in Kodak D-19 developer, washed in tap water, and fixed with Kodak fixer.

## Brain data analysis

Brightfield photomicrographs of the Nissl stained brain sections were made with a Leica DM6000B digital microscope (Leica Microsystems), equipped with a Leica DFC420 5 megapixel CCD digital color camera. The Nissl sections were manually co-registered with the in situ hybridization autoradiograms of parallel brain sections to reliably determine the location of brain areas, such as the robust nucleus of arcopallium (RA) and the preoptic area (POA), which were difficult to distinguish from the autoradiograms directly. The data analysis of mRNA expression patterns was carried out similarly as described in Voigt et al. (2009). Autoradiograms were scanned with an Epson scanner using SilverFast Ai software as 16 bit grey values and with a resolution of 2400 dpi for later analysis in ImageJ. The system was calibrated by scanning a calibrated optical density step tablet (part #T2115, Stouffer Graphic Arts Equipment Co., Mishawaka, USA) and a calibration curve was calculated based on the Rodbard function in ImageJ. All autoradiogram images were saved in ImageJ with this calibration. HVC volumes were estimated based on androgen receptor expression (see also (Gahr and Metzdorf 1997; Fusani et al. 2000). For each androgen receptor-labeled brain section we delineated HVC, summed the area measurements and multiplied them with 100 $\mu$m (interval between sections). Optical densities of androgen receptor, estrogen receptor $\alpha$ and aromatase mRNA expression levels in the preoptic area were measured in two different ways using an ellipsoid with fixed dimensions for all sections and individuals, and again using an ellipsoid covering most of the stained area (therefore with changing size between individuals). To control for background staining the optical density in a control area just adjacent to the preoptic area was subtracted from the value for receptor expression. Optical density measures were averaged across all sections that were labeled for the respective mRNA. All measurements were carried out blind to the seasonal group of the bird.

## Statistical analysis

Data were analyzed in R Development Core Team (2009). Hormonal data were analyzed using linear models. In a first step we used a priori set contrasts to compare the breeding substages (incubation, nestlings, and fledglings) and the nonbreeding period with the breeding substage territory establishment. In a second step we only compared testosterone levels of males caught in different phases within the breeding season (first and second brood vs. territory establishment). Spontaneous song and brain data were only collected during breeding territory establishment and nonbreeding territoriality. Spontaneous song data were tested for seasonal differences using t-tests and were transformed, if necessary, to meet assumptions of equality and normality of variances. Brain data did not meet these assumptions and were, therefore, tested for seasonal differences using non-parametric Mann-Whitney-U-tests.

# Results

## Seasonal testosterone profile

Testosterone levels of males caught at various times of the year (see next sentence) differed significantly ($F_{5,193}$ = 34, p < 0.0001, Fig. 2.1). A priori set contrasts revealed that testosterone levels of males during all other phases of the life cycle were significantly lower than during territory establishment (incubation: t = −2.3, p = 0.02, nestlings (first and second brood combined): t = −6.4, p < 0.0001; fledglings (first and second brood combined): t = −8.5, p < 0.0001; molt: t = −3.1, p = 0.002; nonbreeding territoriality: t = −11.7, p < 0.0001). Within the breeding life-cycle stage, testosterone levels also differed significantly ($F_{2,178}$ = 99, p < 0.0001). Testosterone levels of males caught during their first brood did not significantly differ from those of males caught during territory establishment (t = −1.8, p = 0.08), but males caught during the second brood had significantly lower testosterone levels than males caught during territory establishment (t = −13.3, p < 0.0001). During early breeding in April testes were fully developed (volume of the left testis (mean ± 95% CI): 76.1 ± 14.0 mm$^3$, n = 8) and significantly larger than during nonbreeding territoriality in September, when testes were completely regressed (0.5 ± 0.5 mm$^3$, n = 8, U = 0, p = 0.0002).

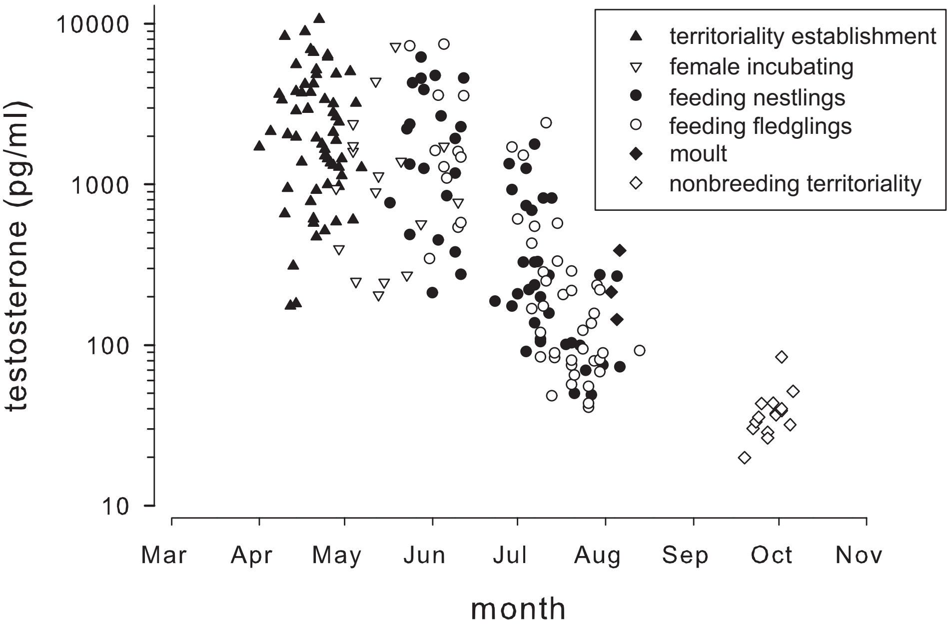

**Fig. 2.1 Post-capture testosterone levels (ng/ml) of male black redstarts caught during different life-cycle stages**. Testosterone levels are presented on a log-scale. Territory establishment: n = 60, male testosterone levels during female incubation: n = 17, nestlings first brood: n = 21, fledglings first brood: n = 12, from July on: nestlings second brood: n = 33, fledglings second brood: n = 38, molt: n = 3, nonbreeding territoriality: n = 15. Most birds caught after the 21 July had already initiated molt of the wing feathers even if they were still feeding young. Each individual is represented only once.

## Behavioral response to simulated territorial intrusions

Territorial male black redstarts did not significantly differ between life-cycle stages in the time they spent within 5 m of a simulated territorial intruder, the time they spent with feathers fluffed in response to a simulated territorial intruder or the percentage of individuals attacking a simulated territorial intruder (Fig. 2.2, Apfelbeck and Goymann, 2011). However, a significantly higher percentage of individuals responded to a territorial intruder with song during the breeding life-cycle stages than during nonbreeding territoriality (Fig. 2.2, Apfelbeck and Goymann, 2011).

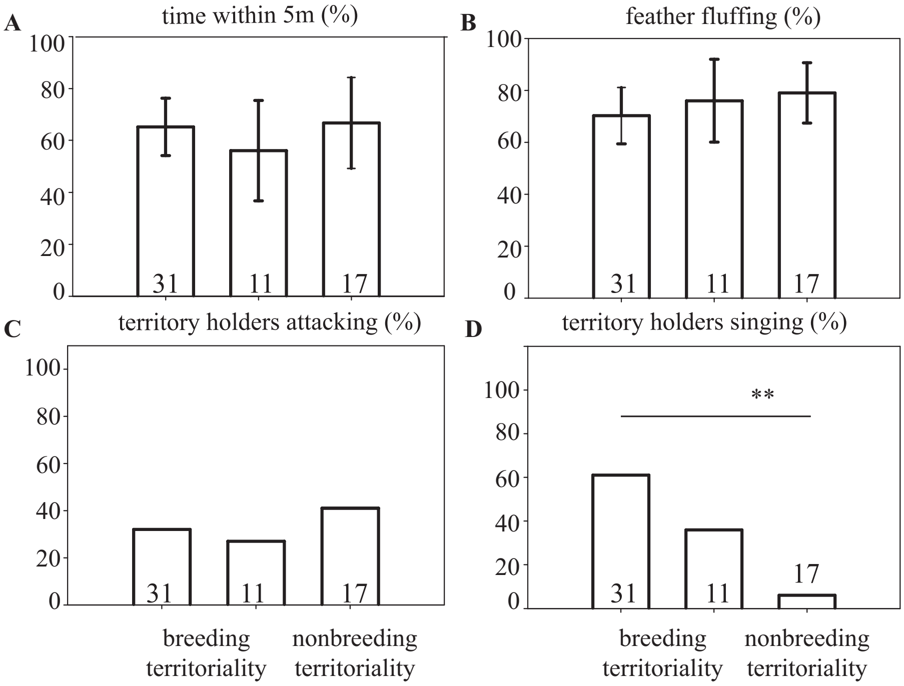

**Fig. 2.2 Time spent within 5 m (as % of time seen, A), time spent feather fluffing (as % of time seen, B), number of individuals attacking (%, C) and number of individuals singing (%, D) in response to a simulated territorial intruder.** Horizontal axes depict percent. Breeding territoriality is split into territory establishment (bars to the left) and feeding of nestlings or fledglings of the first brood (bars in the middle). Bars represent means ± 95% CI, numbers within bars represent sample sizes. Asterisks indicate significant differences between breeding stages (**$p < 0.01$). For statistics and a description of the territorial intrusion experiments see (Apfelbeck and Goymann, 2011).

## Spontaneous song: differences between life-cycle stages

Territorial male black redstarts sang significantly more songs with shorter pauses between songs during nonbreeding than during breeding (Table 2.1). Songs during breeding had more elements in part A and C than those sang during nonbreeding (Table 2.1). All other structural song parameters did not differ significantly between life-cycle stages (Table 2.1).

**Table 2.1 Comparison of song parameters during bouts of spontaneous song between breeding and nonbreeding territoriality**. Significant results are highlighted in bold.

| | | | Mean ± 95% CI | |
|---|---|---|---|---|
| Song parameter | t - Statistic (df = 30) | p | Breeding (n = 20) | Non-breeding (n = 12) |
| **Song rate (songs/min)** | **3.9** | **0.0005** | **3.1 ± 1.1** | **6.1 ± 1.5** |
| **Pauses between songs (s)** | **−3.2** | **0.004** | **11.3 ± 5.1** | **6.0 ± 1.8** |
| Duration A (s) | 0.7 | 0.5 | 0.9 ± 0.1 | 0.9 ± 0.1 |
| Duration B (s) | 0.6 | 0.6 | 0.9 ± 0.2 | 0.9 ± 0.2 |
| Duration C (s) | −1.8 | 0.09 | 0.8 ± 0.1 | 0.7 ± 0.1 |
| Duration pause A-B (s) | −0.4 | 0.7 | 1.3 ± 0.4 | 1.2 ± 0.5 |
| **No. of elements in A** | **2.0** | **0.05** | **8.4 ± 1.5** | **6.9 ± 1.1** |
| **No. of elements in C** | **2.8** | **0.009** | **6.8 ± 1.1** | **5.2 ± 1.0** |
| Max frequency A (kHz) | −0.6 | 0.6 | 6.2 ± 0.2 | 6.1 ± 0.2 |
| Freq bandwidth A (kHz) | 0.5 | 0.6 | 3.2 ± 0.3 | 3.3 ± 3.9 |
| Max frequency B (kHz) | 0.1 | 0.9 | 8.2 ± 3.7 | 8.2 ± 2.6 |
| Freq bandwidth B (kHz) | 0.4 | 0.7 | 6.0 ± 3.9 | 6.1 ± 3.3 |
| Max frequency C (kHz) | 1.7 | 0.09 | 5.9 ± 2.7 | 6.2 ± 2.2 |
| Freq bandwidth C (kHz) | 1.9 | 0.07 | 3.2 ± 3.5 | 3.5 ± 3.5 |

## Expression patterns of androgen receptor (AR), estrogen receptor (ER $\alpha$ ) and aromatase

### *Telencephalon*

Dense androgen receptor mRNA staining was found in the song control nuclei HVC and in the lateral and medial nucleus magnocellularis (lMAN and mMAN, Fig. 2.3, Table 2.2) independent of life-cycle stage. However, in contrast to previous studies in other songbirds, androgen receptor expression in the robust nucleus of arcopallium (RA) was weak (Fig. 2.4). Instead, RA was surrounded by a band of androgen receptors, as demonstrated by relatively dense androgen receptor expression that was contained to the caudal arcopallium and did not extend rostrally (Fig. 2.4). Weak estrogen receptor $\alpha$ mRNA expression was only found in the so-called paraHVC (Table 2.2, see Gahr and Metzdorf, 1997 for a description of this region). Aromatase expression was not found in any of the song control nuclei (Table 2.2). However, similar to other songbirds, dense staining for aromatase mRNA occurred in the caudo-medial nidopallium (NCM, Table 2.2, (Shen et al. 1995). Similar to canaries, aromatase and estrogen receptor $\alpha$ expression in NCM did not overlap (Metzdorf et al. 1999), but aromatase and androgen receptor expression did. Estrogen receptor $\alpha$ in NCM was specifically expressed around the lateral ventricle.

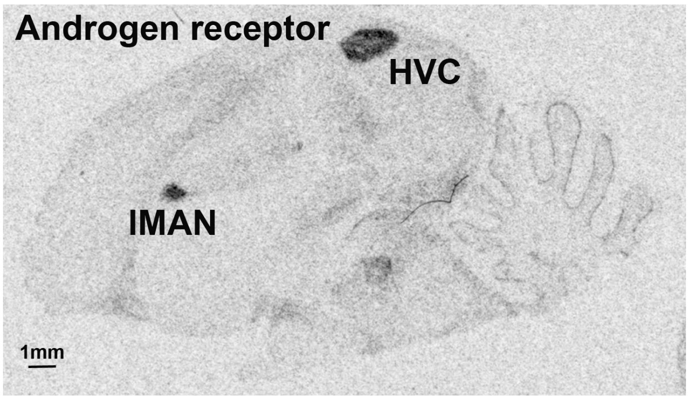

**Fig. 2.3 Example of androgen receptor mRNA expression in two song control nuclei during the early breeding season**. HVC: proper name, lMAN: lateral nucleus magnocellularis.

**Table 2.2 Brain areas in the telencephalon and diencephalon expressing androgen receptor (AR), estrogen receptor (ERα) or aromatase (ARO) depending on life-cycle stage**. lMAN, lateral nucleus magnocellularis; mMAN, medial nucleus magnocellularis; NCM, caudo-medial nidopallium; POA, pre-optic area; PMH, nucleus posterioris hypothalami medialis; PLH, nucleus posterioris hypothalami lateralis; BNST, bed nucleus of the stria terminalis; RA, robust nucleus of arcopallium. We visually graded the expression in the different areas into ++ strong expression, + weak expression or − no expression. Only in the preoptic area aromatase expression seemed to differ with life-cycle stage (bold areas).

| Brain area | AR breeding | AR non-breeding | ARO breeding | ARO non-breeding | ER α breeding | ER α non-breeding |
|---|---|---|---|---|---|---|
| **Telencephalon** | | | | | | |
| HVC | ++ | ++ | − | − | ++ | ++ |
| lMAN | ++ | ++ | − | − | − | − |
| mMAN | ++ | ++ | − | − | − | − |
| RA | + | + | − | − | − | − |
| Arcopallium | ++* | ++* | − | − | − | − |
| NCM | ++ | ++ | ++ | ++ | ++ | ++ |
| **Diencephalon** | | | | | | |
| POA | ++ | ++ | **++** | **+** | ++ | ++ |
| PMH | ++ | ++ | ++ | ++ | − | − |
| PLH | ++ | ++ | ++ | ++ | ++ | ++ |
| Tuberal region | ++ | ++ | + | + | ++ | ++ |
| BNST | ++ | ++ | ++ | ++ | ++ | ++ |

* Expression was restricted to the caudal arcopallium.

## Diencephalon: preoptic and hypothalamic areas

During both breeding and nonbreeding territoriality co-expression of androgen receptor, estrogen receptor α and aromatase was found in the preoptic area (Fig. 2.5, Table 2.2). Androgen receptor, estrogen receptor α and aromatase were also co-expressed in the nucleus lateralis hypothalami posterioris (PLH, Fig. 2.5, Table 2.2), but similar to other species no estrogen receptor α expression was found in the nucleus medialis hypothalami posterioris (PMH, Fig. 2.5, Table 2.2, (Fusani et al., 2000 and Metzdorf et al., 1999). The tuberal region was densely stained for estrogen receptor α and androgen receptor mRNA and weakly stained for aromatase mRNA (Fig. 2.5, Table 2.2). Further staining for androgen receptor, estrogen receptor α and aromatase mRNA was found in the bed nucleus of the stria terminalis (BNST, Table 2.2).

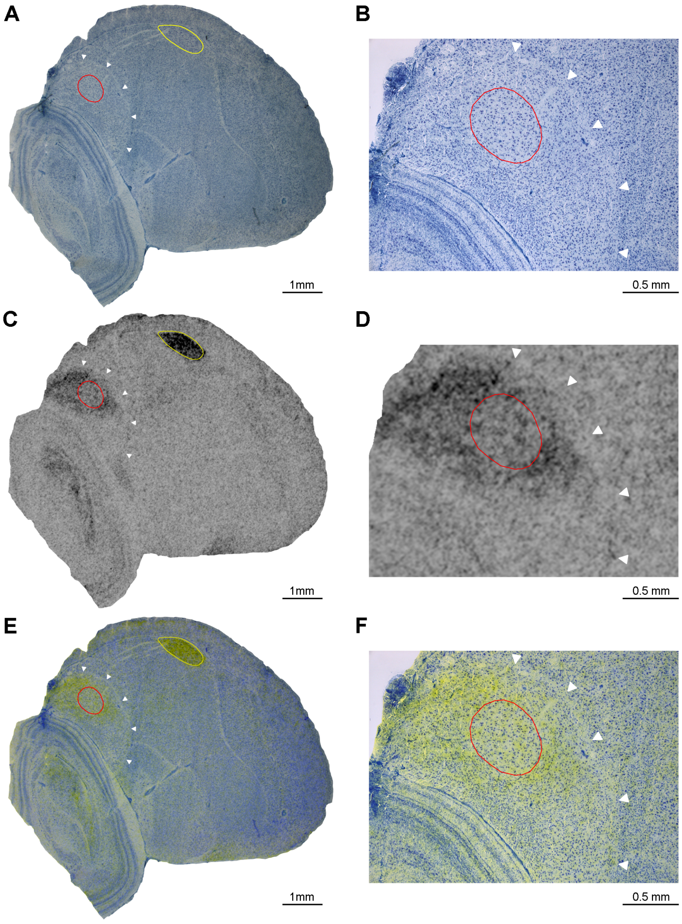

**Fig. 2.4 Androgen receptor expression in the arcopallium and in HVC**. (A, B): Nissl stained sagittal brain sections. C, D: In-situ hybridization autoradiogram for androgen receptor expression in brain sections adjacent to sections shown in (A, B). (E, F): Overlay of the Nissl stained sections (A, B) with sections stained for androgen receptor (C, D). Yellow circles indicate the border of HVC and red circles delineate RA as determined in the Nissl stained sections. (B, D, F) show magnifications of the arcopallium of the sections presented in (A, C, E), and closed arrowheads indicate the dorsal border of the arcopallium.

## HVC volume

The HVC volume determined by androgen receptor expression did not significantly differ between breeding and nonbreeding territoriality (mean ± 95% CI: breeding: 0.6 ± 0.1 mm$^3$, n = 8, nonbreeding: 0.8 ± 0.08 mm$^3$, n = 8; Mann-Whitney U test: U = 50, df = 12, p = 0.06).

## Optical density in the preoptic area

As both methods to measure optical density produced similar results, we only present results for one of the methods (i.e. ellipsoid fitted to area with staining). Aromatase mRNA expression in the preoptic area was significantly higher during breeding territoriality than during the nonbreeding territoriality (U = 45; p = 0.007, n = 8 per season). Expression of estrogen receptor mRNA (U = 12, p = 0.1) and androgen receptor mRNA (U = 26.5, p = 0.9) did not differ significantly between life-cycle stages.

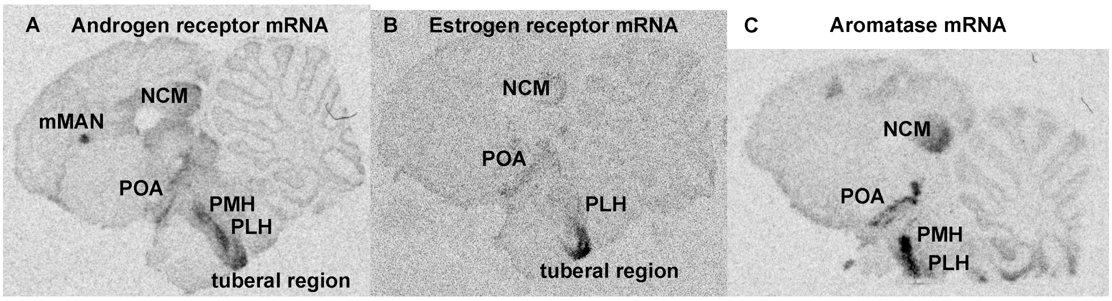

**Fig. 2.5 Example of androgen receptor (A), estrogen receptor (B) and aromatase (C) mRNA expression during breeding territoriality**. mMAN: medial nucleus magnocellularis, NCM: caudo-medial nidopallium, POA: preoptic area, PMH: nucleus posterioris hypothalami medialis, PLH: nucleus posterioris hypothalami lateralis.

# Discussion

## Non-vocal territorial behavior and testosterone

In black redstarts, the expression of non-vocal territorial behaviors did not correlate with differences in testosterone levels and aromatase expression in the preoptic area. Because males vigorously defended territories both during breeding and during fall nonbreeding while testosterone levels and aromatase expression in the preoptic area were higher during breeding than during fall nonbreeding territoriality. Thus, territorial behavior does not seem to be maintained by an increased sensitivity for sex steroids during nonbreeding; i.e. upregulation of aromatase (higher levels of aromatase during nonbreeding compared to breeding) metabolizing low levels of testosterone (Fusani et al. 2007). Instead the increased expression of aromatase during breeding in the preoptic area is similar to other temperate-zone bird species (Foidart et al. 1998; Silverin et al. 2000; Wacker et al. 2010) and suggests that also in black redstarts the preoptic area plays a role in estrogen-dependent regulation of male reproductive behavior (Ball and Balthazart 2004; Balthazart et al. 2010).

Similar to black redstarts, song sparrows aggressively defend territories in a nonbreeding context and have higher testosterone levels and higher aromatase expression and activity in the preoptic area during breeding than during nonbreeding territoriality (Wacker et al. 2010). Although song sparrows respond to a simulated territorial intruder during all life-cycle stages, the response is much reduced during molt (Wingfield and Hahn 1994). Wacker et al. (2010) propose that in song sparrows aggressive behavior is regulated by aromatase in the ventromedial hypothalamus because a weak response to a territorial intruder during molt correlates with low expression of aromatase in that area. In addition, aromatase inhibitors reduced aggression during nonbreeding in song sparrows and the aggressive response was rescued by estrogen treatment (Soma et al. 2000a,b). However, although we have not assessed aggressive behavior and receptor expression during molt in male black redstarts we suggest that in redstarts non-vocal territorial behaviors are independent of sex steroidal control as treatment with an androgen receptor blocker and an aromatase inhibitor during breeding did not reduce non-vocal territorial behaviors (Apfelbeck et al. 2013a).

## Vocal behavior, brain and testosterone

Similar to black redstarts male canaries spontaneously sing more syllable types with trills (Leitner et al. 2001; Voigt and Leitner 2008) and male song sparrows sing trills with more elements (Smith et al. 1997) during breeding than during nonbreeding. These changes have been suggested to be mediated by testosterone as they follow seasonal changes in plasma testosterone levels (Smith et al. 1997; Leitner et al. 2001; Voigt and Leitner 2008). More specifically, these seasonal changes in song structure seem to be regulated by estrogen-dependent mechanisms in HVC in canaries (Gahr and Metzdorf 1997; Fusani et al. 2003; Rybak and Gahr 2004; Fusani and Gahr 2006) and by testosterone-dependent seasonal changes in HVC size in song sparrows and Gambel's white-crowned sparrows (*Zonotrichia leucophrys gambelii*, (Troy Smith et al. 1995; Smith et al. 1997; Soma et al. 1999; Fraley et al. 2010). The expression pattern of androgen receptor, estrogen receptor and aromatase mRNA of male black redstarts is similar to the expression pattern found in canaries and other songbirds (but see expression in the caudal arcopallium, Fig. 2.4, (Gahr and Metzdorf 1997; Brenowitz 2008; Fraley et al. 2010). Thus, similar mechanisms may regulate seasonal changes in song. However, in male black redstarts HVC size and sensitivity for androgens or estrogens do not seem to differ between breeding and nonbreeding territoriality. Furthermore, in another experiment treatment of males with an antiandrogen and an aromatase inhibitor during breeding and fall nonbreeding did not reduce the number of repetitive elements produced (Apfelbeck et al. 2012). This suggests that the seasonal changes in the production of repetitive elements in the song are not directly induced by sex steroid-dependent changes in nucleus HVC in black redstarts. This demonstrates that although the song system seems to be fairly conserved across songbirds, the mechanistic control of song production may still differ between species and more studies especially on wild birds under natural conditions are necessary to understand this diversity of mechanisms in the production of song.

In contrast to the seasonal decrease in repetitive elements in spontaneous song, spontaneous song output was even higher during nonbreeding than during breeding territoriality. During breeding males were sampled during a longer period of time than during nonbreeding and some females had already started to incubate. Males of these females probably decreased their song rate (Landmann 1996). A high song rate during the fall nonbreeding territorial phase also suggests that spontaneous song rate in male black redstarts is independent of testosterone. This is in contrast to most other temperate-zone songbirds in which song rate decreases steeply after breeding and in concert with plasma testosterone levels (reviewed in (Ball et al. 2002)). However, in contrast to spontaneously produced song, the likelihood of song produced in response to a territorial intruder was significantly reduced during nonbreeding territoriality (Fig. 2.2). This suggests that in male black redstarts seasonal changes in testosterone do not regulate song output in general, but in a context-dependent way. Similarly, European starlings (*Sturnus vulgaris*) sing at a high rate also during nonbreeding, but respond to females with an increase in song rate only during breeding (Riters et al. 2000). This sexually motivated change in seasonal song production has been suggested to be facilitated by testosterone through indirect effects outside the song control system: it is correlated with high aromatase activity (Riters et al. 2000; Pintér et al. 2011) and immediate early gene expression in the preoptic area during breeding (Heimovics and Riters 2005). Thus, the preoptic area supposedly does not only play a major role in the regulation of sexual behavior per se, but also the expression of other sexually motivated behaviors (Riters 2012).

## Testosterone and the regulation of non-vocal and vocal territorial behaviors

Combined evidence from this and previous studies suggests that testosterone and its metabolites regulate territorial behavior in black redstarts only partly and in a complex context-dependent manner. The non-vocal territorial response and spontaneously produced song seem to be independent of the control by sex steroids (Apfelbeck and Goymann, 2011, Fig. 2.2, Table 2.3). However, song produced in an agonistic context is probably facilitated by sex steroids (Apfelbeck and Goymann, 2011, Fig. 2.2, Table 2.3). These findings are similar to results obtained from two tropical songbird species. In staged male-male encounters during nonbreeding, captive spotted antbirds (*Hylophylax n. naevioides*) only responded with song when they were implanted with testosterone and during breeding, males implanted with blockers reduced the number of songs during such encounters (Hau et al. 2000). Costa Rican rufous collared sparrows (*Zonotrichia capensis costaricensis*) reacted ‑ similar to male black redstarts ‑ with equal intensity to a territorial intruder across seasons, however, they responded with significantly fewer songs during nonbreeding (Addis et al. 2010). In another rufous-collared sparrow population, *Zonotrichia capensis*, territorial behavior is completely independent of testosterone (Moore et al. 2004). Furthermore, these data corroborate similar findings in other bird species that defend territories outside the breeding season: males can respond to intruders and defend a territory even with low circulating levels of testosterone (Burger and Millar 1980; Logan and Wingfield 1990; Schwabl and Kriner 1991; Wingfield 1994a; Soma and Wingfield 1999; Canoine and Gwinner 2002; Landys et al. 2010; Hau and Beebe 2011). In addition to its signal function to other males, song may provide relevant cues for females during breeding. In most songbirds, song is not only important for territory defense, but also to attract and stimulate mates. In many species, including black redstarts (Landmann 1996), during the breeding season song rate is highest when females are fertile (Mace 1987; Gil and Gahr 2002). Furthermore, it has been shown that females pay attention to the vocal output in singing interactions of males during agonistic encounters (Otter et al. 1999; Ballentine et al. 2004). Thus, seasonal changes in social context-dependent song output may be sexually motivated and selected and may be facilitated by the conversion of testosterone to estradiol in the preoptic area.

**Table 2.3 Summary of physiological, morphological and behavioral results comparing breeding and nonbreeding territoriality of male black redstarts.**

| | Breeding | Nonbreeding |
|---|---|---|
| **Endocrine parameters** | | |
| Testosterone | High | Low |
| Testes | Large | Regressed |
| **Response to STI** | | |
| Non-vocal | Strong | Strong |
| Probability of song | High | Low |
| **Spontaneous song** | | |
| Song rate | Medium | High |
| Song structure | More elements in A and C | Fewer elements in A and C |
| **Brain anatomy** | | |
| HVC | Large | Large |
| Aromatase pre-optic area | Strong | Weak |

## Conclusions

Our studies show that the relationship between testosterone and territorial behavior in male black redstarts is complex: testosterone does not seem to modulate non-vocal territorial behaviors, but the hormone may be involved in the regulation of context-dependent song through aromatization in the preoptic area. Our studies on black redstarts also suggest that there is no one unique mechanism by which sex steroids regulate territorial and song behavior in songbirds. Rather there seems to be a variety of solutions to the control of year round territoriality that we are just beginning to understand.

# Acknowledgements

We are grateful to Johanna Stegherr, Alexander Weber, Lisa Trost, Benjamin Wasmer and Stefan Leitner for their help in the field, to Ingrid Schwabl and Monika Trappschuh for support in the hormone analysis, to Christina Wolf for conducting the in situ-hybridizations and to Cornelia Voigt and Harriet Windley for their help in the analysis of the in situ-hybridization results. We also want to thank two anonymous reviewers for valuable comments on a previous version of this manuscript.

# CHAPTER 3 Life-history and hormonal control of aggression in black redstarts: Blocking testosterone does not decrease territorial aggression, but changes the emphasis of vocal behaviors during simulated territorial intrusions

*Apfelbeck B, Mortega KG, Kiefer S, Kipper S, Goymann W. 2013 Frontiers in Zoology, 10:8*

# Abstract

Many studies in behavioral endocrinology attempt to link territorial aggression with testosterone, but the exact relationship between testosterone and territorial behavior is still unclear and may depend on the ecology of a species. The degree to which testosterone facilitates territorial behavior is particularly little understood in species that defend territories during breeding and outside the breeding season, when plasma levels of testosterone are low. Here we suggest that species that defend territories in contexts other than reproduction may have lost the direct regulation of territorial behavior by androgens even during the breeding season. In such species, only those components of breeding territoriality that function simultaneously as sexually selected signals may be under control of sex steroids. We investigated black redstarts (*Phoenicurus ochruros*), a species that shows periods of territoriality within and outside of the breeding season. We treated territorial males with an anti-androgen and an aromatase inhibitor during the breeding season to block both the direct and indirect effects of testosterone. Three and ten days after the treatment, implanted males were challenged with a simulated territorial intrusion. The treatment did not reduce the overall territorial response, but it changed the emphasis of territoriality: experimental males invested more in behaviors addressed directly towards the intruder, whereas placebo-treated males put most effort into their vocal response, a component of territoriality that may be primarily directed towards their mating partner rather than the male opponent. In combination with previous findings, these data suggest that overall territoriality may be decoupled from testosterone in male black redstarts. However, high levels of testosterone during breeding may facilitate context dependent changes in song.

# Introduction

In a reproductive context, testosterone and its metabolite estradiol are considered major hormones facilitating territorial behavior and the associated vocalizations in a wide range of male vertebrates [e.g. (Lincoln et al. 1972; Wingfield et al. 1990). Particularly in birds, seasonal peaks in testosterone closely match periods of intense male-male competition for territories and mates (Wingfield et al. 1990). Furthermore in songbirds, testosterone and estradiol play an important role in the activation of song during the breeding season [reviewed in (Ball and Balthazart 2010).

Sex steroids, such as testosterone and estradiol, orchestrate physiological, morphological and behavioral changes important for reproduction [e.g. (Lincoln et al. 1972). A close link between the expression of territoriality and testosterone ensures that this behavior is expressed only in the appropriate breeding life-history context, as maintaining high levels of territorial aggression can be energetically costly and may impair survival (Dufty 1989; Wingfield et al. 2001). However, for example in songbirds, it is quite common that males also defend territories outside a breeding context, when testes are regressed and testosterone levels are low (Table 3.1, (Wingfield 1994a; Landys et al. 2010)). When behavior is expressed over a longer period of time, throughout the year, or in different life-history stages its control may be decoupled from hormones [e.g. 4, see also (Landys et al. 2013). Hence, when territorial behavior occurs in many life-history contexts (i.e. most time of the year) it may be independent of testosterone. Only few species have been studied in this regard: these studies suggest that testosterone plays a role in the regulation of breeding season territoriality also in species that defend territories outside a breeding context (Table 3.1). However, the degree to which testosterone facilitates territoriality appears to differ between species. The following three scenarios might explain these differences.

First, in some species the intensity of territorial aggression differs between breeding and non-breeding contexts with males expressing only low levels of territorial aggression outside the breeding season. This low-intensity territorial behavior may be independent of testosterone, but testosterone may intensify territoriality in a breeding context (e.g. mountain spiny lizards, *Sceloporus jarrovi* (Moore 1986, 1988), European nuthatches, *Sitta europea*, (Landys et al. 2013).

Second, testosterone may facilitate territoriality in breeding and non-breeding contexts, but the source of testosterone may depend on the life-history stage. Song sparrows, *Melospiza melodia*, show similar levels of territorial aggression during the breeding and the non-breeding season (Wingfield 1994a). In the non-breeding season, when the circulating testosterone levels of song sparrows are low, testosterone may be produced locally in the brain by conversion of non-gonadal dehydroepiandrosterone (DHEA, (Soma et al. 2000a; Soma and Wingfield 2001).

Third, during the breeding season sex steroids may activate exclusively those components of territorial behavior that are relevant in the breeding context. Territoriality consists of a variety of behaviors including vocalizations (song, calls), spatial behaviors, threat displays and direct aggression. Similar to courtship displays (Fusani et al. 2001) these different components may be facilitated by different (hormonal) pathways (Schwabl and Kriner 1991; Van Duyse et al. 2002; Sperry et al. 2010). During the breeding season, testosterone may specifically activate those aspects of the territorial response that also involve signaling to females. For example, in the grey partridge, *Perdix perdix,* testosterone manipulations affected the quality of the rusty gate call and its salience for females (Beani and Dessì-Fulgheri 1995; Beani et al. 1995). The same pattern may account for the results on territorial behavior found in most of the bird species studied so far as a strong overall effect of testosterone on territorial behavior has been the exception rather than the rule (Table 3.1): birds implanted with androgen receptor blockers (and aromatase inhibitors) did not lose their territories. In most cases the treatment only reduced some aspects of the territorial behavior or had no effect at all on any of the behaviors measured. In addition, in studies where blocking the action of testosterone had an effect on territorial behavior, this effect was usually found during the breeding life-history stage, but not outside a breeding context (Table 3.1).

The aim of this study was to investigate the role of testosterone and its metabolite estradiol in the regulation of breeding season territoriality in short-distance migratory male black redstarts, *Phoenicurus ochruros* (Fig. 3.1). We hypothesize that in this species only some aspects of territoriality may be facilitated by testosterone during breeding, thus supporting the third scenario described above. Males of this species are highly territorial in spring when their testosterone levels are elevated, but also in autumn, just before migration, when testosterone is basal (Apfelbeck and Goymann 2011). In both life-history contexts they express androgen and estrogen receptors and aromatase in brain areas that are relevant for song, sexual and aggressive behaviors (Apfelbeck et al. 2013b). Unlike some other species (reviewed in (Goymann 2009)), male black redstarts do not increase testosterone during agonistic encounters with other males or during simulated territorial intrusions (STI) with a male decoy (Apfelbeck and Goymann 2011; Apfelbeck et al. 2011).

**Table 3.1 Effects of androgen receptor blocker and/or aromatase inhibition treatment on territoriality and aggression in different species of birds**

| **Species** | **Treat** | **Days** | **Song output** | **Song str.** | **Calls** | **App. lat.** | **Closest app.** | **Spent close** | **Lat. attack** | **Flights chases attacks pecks** | **Threat display** | **Loss** | **Reference** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Breeding season** | | | | | | | | | | | | | |
| Songbirds | | | | | | | | | | | | | |
| European stonechat | AR/Aro | 7-17 | | | | | ↑ | | ↑ | | | no | Canoine et al. 2002,<br>Marasco et al. 2011 |
| European robin | AR | 8-14 | no | | | no | no | no | | | | no | Schwabl et al. 1991 |
| | | 18-25 | no | | | ↑ | no | no | | | | no | |
| Song sparrow (pre-breeding) | AR | 18 | no | | | no | no | no | | ↓ | | no | Sperry et al. 2010 |
| Song sparrow | AR | 18 | no | | | no | no | no | | no | | no | Sperry et al. 2010 |
| Song sparrow | Aro | 24hrs | no | | | no | no | no | | no | | no | Soma et al. 2000 |
| | | 8-10 | no | | | no | no | no | | no | | no | |
| Red-winged blackbird(*) (polygynous) | AR/Aro | 2-5 | no [1] | | no | | | | | no | | no [2] | Beletsky et al. 1990 |
| Red-winged blackbird(*) | AR | 4-12 | no | | no | | | | | no | | no | Beletsky et al. 1990 |
| Great tit | AR/Aro | 2-5 | ↓ [3] | no [4] | | | | | | | | no | Van Duyse et al. 2005 |
| Spotted antbird | AR/Aro | 8 | ↓ [5] | | less snarls | | | | | | no | lab | Hau et al. 2000 |
| Rufous-collared sparrow | AR/Aro | 7-13 | no | | | no | no | no | | no | | no | Moore et al. 2004 |
| House sparrow (x) | AR | 7-10 | | | | | | | | | | no[6] | Hegner et al. 1987 |
| Non-songbirds | | | | | | | | | | | | | |
| Corncrake | AR | 2 | | | ↓ | | | ↓ | | | | no | Ręk et al. 2011 |
| Bobwhite quail | AR | 20 | | | | | | | | ↓ | | lab | Vleck et al. 1993 |
| Screech owl | AR/Aro | 7-14 | no | | | | no | ↓ | | | | no | Herting et al. 1997 |
| Japanese quail (#) | Aro | 1-10 | | | | | | | | ↓ | | lab | Schlinger et al. 1990 |
| Japanese quail (#) | AR | 1-10 | | | | | | | | no | | lab | Schlinger et al. 1990 |
| **Non-breeding season** | | | | | | | | | | | | | |
| Songbirds | | | | | | | | | | | | | |
| European stonechat | AR/Aro | 7-17 | | | | | no | | no | | | no | Canoine et al. 2004 |
| European robin | AR | 6 | no | | | no | no | no | | | | no | Schwabl et al. 1991 |
| | | 31-39 | no | | | no | no | no | | | | no | |
| Song sparrow | Aro | 24hrs | no | | | no | ↑ | no | | ↓ | | no | Soma et al. 2001 |
| | | 9-12 | ↓ | | | ↑ | ↑ | ↓ | | ↓ | | no | Soma et al. 2000 |
| Song sparrow | AR/Aro | 7 | no | | | no | no | no | | no | | no | Soma et al. 1999 |

| **Species** | **Treat** | **Days** | **Song output** | **Song str.** | **Calls** | **App. lat.** | **Closest app.** | **Spent close** | **Lat. attack** | **Flights chases attacks pecks** | **Threat display** | **Loss** | **Reference** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
|  |  | 30 | ↓ |  |  | ↑ | ↑ | ↓ |  | ↓ |  | no |  |
| Spotted antbird | AR/Aro | 8 | no |  | no |  |  |  |  |  | no | lab | Hau et al. 2011 |
| Red-winged blackbird(+) | AR | 1-15 |  |  |  |  |  |  |  | ↓ |  | yes | Searcy et al. 1980 |
| Non-songbirds |  |  |  |  |  |  |  |  |  |  |  |  |  |
| Red grouse | AR/Aro | 14-21 |  |  | no |  |  |  |  |  |  | no | Mougeot et al. 2005 |
| Screech owl (n = 2 – 3) | AR/Aro | 7-14 | no |  |  |  | no | (↓) |  |  |  | no | Herting et al. 1997 |

"Treat" indicates the type of blocker treatment with either AR (androgen receptors blocked) and/or Aro (aromatase and thus the conversion of androgens to oestrogens blocked). "Song str." = changes in song structure; "app. lat" = latency to approach a decoy; "closest app." = closest approach to a decoy; "spent close" = time spent close to a decoy; "lat. attack" = latency to attack a decoy; "loss" = loss of territory or loss of dominance. All species except spotted-antbirds and rufous-collared sparrows were non-tropical species. Most of the studies assessed territorial behaviour by challenging free-living treated territory owners with simulated territorial intrusions, except:

(*) naturally occurring territorial aggression,

(x) observation of nest site defence.

(+) lab studies that quantified aggression and dominance between group-housed males.

(#) lab study assessing locomotor activity and pecking rate in response to a stimulus female behind glass.

Numbers in superscript refer to:

1.More vocalizations in general.

2.Some males lost parts of their territories.

3.The likelihood of dawn song was reduced.

4.There was no effect on song duration or repertoire size.

5.They sang less spontaneous song, less song towards females and during STIs.

6.Defence of nest site decreased in AR inhibited individuals, but no loss.

If there was an effect of the treatment, upward arrows indicate that the respective behaviour increased (↑), while downward arrows indicate a decrease in the behaviour (↓). Because of major methodological differences we do not present the study by Archawaranon and Wiley (1988, white-throated sparrows, Aro and testosterone treatment combined) in the table.

**Fig. 3.1 Adult male black redstart during breeding**

Accumulating evidence suggests that in this species non-vocal territorial behaviors are independent of testosterone while song output and structure are regulated by testosterone or its metabolites. In black redstarts males responded equally aggressive to a simulated territorial intruder during breeding and non-breeding, but were less likely to sing in response to the intrusion during non-breeding (Apfelbeck and Goymann 2011). Furthermore, structural changes in the song in response to simulated territorial intruders seem to depend on testosterone or estradiol in the breeding life-history stage (Apfelbeck et al. 2012). These song structures are probably indicative of male quality or the male`s ability and/or motivation to defend a territory as they are characteristic of adult males` song compared to song of yearling males (Cucco and Malacarne 1999). Adult males usually have better territories and a higher breeding success than yearling males (Landmann and Kollinsky 1995b). Furthermore, these song structures were enhanced in the agonistic context (Apfelbeck et al. 2012). Based on these findings we hypothesized that the territorial behavior as such should be decoupled from the control of sex steroids. Only some components of territoriality (e.g. song structure) that are particularly relevant in a mating and breeding context should be influenced by sex steroids.

We implanted male black redstarts with the anti-androgen flutamide (Flut) and the aromatase inhibitor letrozole (Let) and challenged them with a simulated territorial intrusion (using a mounted decoy and audio-playback of black redstart song). As we were interested in the activational effects of testosterone on territorial behavior, we tested males already 3 days after implantation. However, as in some studies effects of anti-androgen and aromatase inhibition became only apparent after a longer period of time (Schwabl and Kriner 1991; Soma et al. 1999), we challenged males a second time 10 days after implantation. We predicted that the ability of Flut/Let-treated males to defend a territory should not differ from that of control males during the breeding season. Further, we predicted that the intensity of non-vocal territorial behaviors should not differ between placebo- and Flut/Let-implanted males. However, based on our previous findings males implanted with Flut/Let should invest less into vocal behavior than placebo-implanted males, resulting in differences in the song responses between groups.

# Results

## Territory maintenance

All placebo- and Flut/Let-implanted males retained their territories during the period when the Flut/Let treatment was effective (~3 weeks). In fact, most of the males, regardless of treatment, still defended the same territory during autumn, i.e. 6 months after the experiment, before they migrated to their wintering grounds (placebo: 9 out of 10, Flut/Let: 8 out of 10).

## Non-vocal behavior during the STI

Flut/Let and placebo-implanted males did not differ significantly in the time they spent within 5 m of the decoy or in the time they fluffed their feathers. However, for both behaviors day of STI had a significant effect in Flut/Let-, but not in placebo- implanted males: Flut/Let-implanted males spent less time within 5 m of the decoy and less time with their feathers fluffed during the STI on day 10 than during the STI on day 3 (5 m: treatment: $F_{1,18} = 0.04$, $p = 0.8$; day: $F_{1,15} = 1.6$, $p = 0.2$; interaction: $F_{1,15} = 5.1$, $p = 0.04$; feather fluffing: treatment: $F_{1,18} = 1.8$, $p=0.2$, day: $F_{1,15}=12.9$, $p = 0.003$, interaction: $F_{1,15} = 7.5$, $p = 0.02$, Fig. 3.2A and B). However, while the two slopes differed significantly, Flut/Let- and placebo implanted males did not significantly differ on day 3 (5 m: $t = 0.36$, $df = 17$, $p = 0.7$; feather fluffing: $t = 0.6$, $df = 16$, $p = 0.6$) and day 10 (5 m: $t = - 0.7$, $df = 18$, $p = 0.5$; feather fluffing: $t = - 2.0$, $df = 14$, $p = 0.06$, Fig. 3.2A and B).

The latency to approach the decoy, the number of head nods and the flights over the decoy did not differ between placebo- and Flut/Let-implanted males (approach latency: treatment: $F_{1,18} = 0.15$, $p = 0.7$, day: $F_{1,16} = 0.14$, $p = 0.7$; interaction: $F_{1,16} = 0.37$, $p = 0.6$; head nods: treatment: $F_{1,18} = 0.1$, $p = 0.7$, day: $F_{1,15} = 3.0$, $p = 0.1$, interaction: $F_{1,15} = 2.0$, $p = 0.2$; flights: treatment: $F_{1,18} = 1.6$, $p = 0.2$; day: $F_{1,16} = 2.3$, $p = 0.1$; interaction: $F_{1,16} = 1.9$, $p = 0.2$). Three males attacked the decoy during the STI on day 3. All of them were implanted with flutamide and letrozole. On day 10 only one of these same males attacked the decoy.

Overall these data suggest that Flut/Let-implanted males showed a reduced non-vocal response on day 10 compared to day 3, because they spent less time close to the decoy and with their feathers fluffed. In placebo-implanted males the non-vocal response did not significantly differ between the STIs on day 3 and 10.

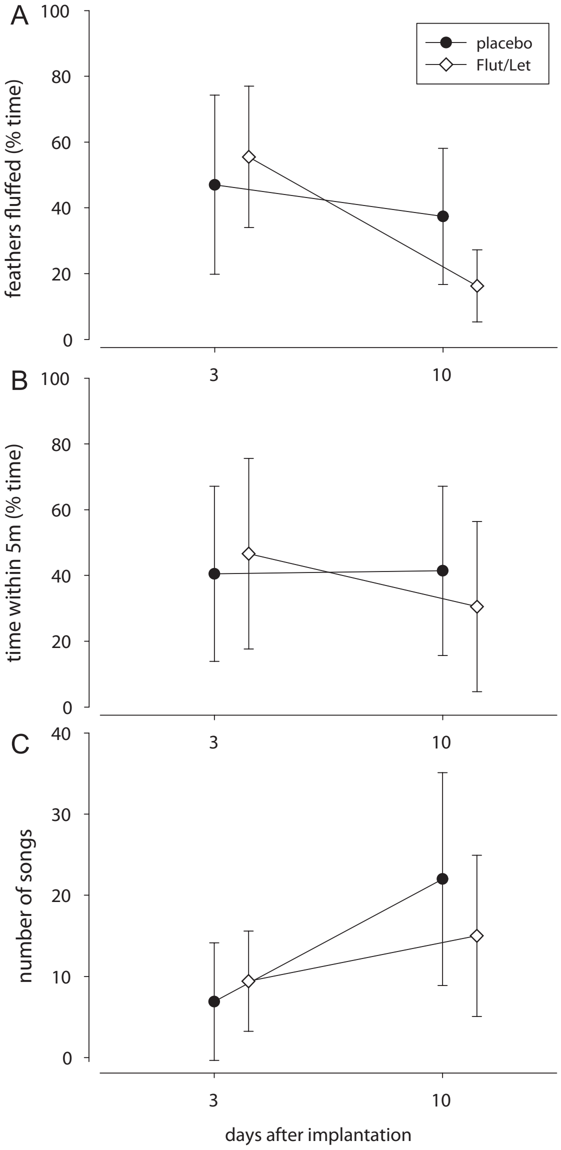

**Fig. 3.2 Non-vocal territorial behaviors (A, B) and number of songs (C).** Behaviors were shown in response to simulated territorial intrusions on day 3 and day 10 after treatment with a placebo or flutamide and letrozole (Flut/Let). Points represent means and error bars represent 95% CI.

## Vocal behavior during the STI

There was no direct effect of the treatment on song output: placebo- and Flut/Let-implanted males did not differ in the number of songs they sang in response to the STI. However, males of both groups sang significantly more songs during the STI on day 10 than on day 3 (treatment: $F_{1,18} = 0.04$, p = 0.8; day: $F_{1,16} = 13.4$, p = 0.002, interaction: $F_{1,16} = 0.4$, p = 0.5; Fig. 3.2C). In measures of song structure (see Fig. 3.3 for a typical black redstart song), we also could not find any direct effect of treatment, but in several measures the treatment interacted with day of STI. Placebo-implanted males sang significantly longer songs on day 10 than on day 3 (Table 3.2, Fig. 3.4A). This was mainly due to a longer pause duration between part A and B on day 10 than on day 3 (Table 3.2, Fig. 3.4B). There was no clear change in Flut/Let- implanted males for both of these measures (Table 3.2, Fig. 3.4). Placebo-implanted males sang part B with a significantly broader frequency bandwidth and with a longer duration on day 3 than on day 10, while there was no clear change in Flut/Let-implanted males (Table 3.2, Fig. 3.5). Furthermore, in placebo-implanted males the relationship between frequency bandwidth and duration of part B was positive, i.e. males that sang parts B with a long duration also sang them with a broad frequency bandwidth (Fig. 3.5). In contrast, this relationship was negative in Flut/Let-implanted males, i.e. birds that sang parts B with larger bandwidths sang these shorter (Fig. 3.5). When controlling for the duration of part A, males of both treatment groups tended to sing it with fewer elements on day 10 than on day 3 (Table 3.2). Thus, both treatment groups sang more songs, but with supposedly lower competitive value during the STI at day 10 than at day 3 as they sang song that was more similar to the song of yearling than to the song of adult males ((Cucco and Malacarne 1999), lower frequency bandwidth and duration of part B in placebo-implanted males, longer songs with longer pause durations between part A and B, less elements in part A). Surprisingly though, the decrease in competitive value of the song from day 3 to day 10 was much more pronounced in placebo- than in Flut/Let-implanted males.

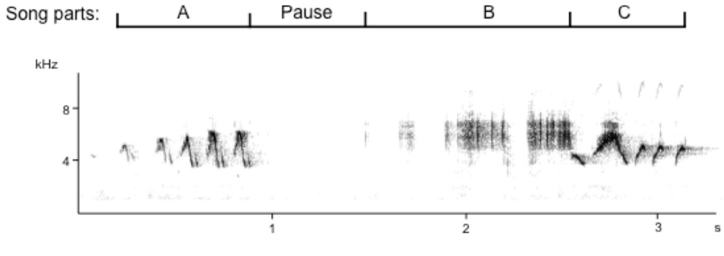

**Fig. 3.3 Spectrogram of one song of a black redstart** (Avisoft-SASLab Pro, sample rate 22.05 kHz, FFT = 256 points, hamming-window, overlap: 50%). Song parts are indicated on top of the spectrogram. Measures analyzed were durations of parts A, B, C, of the total song and the pause duration between A and B; the number of elements of part A and C; the frequency bandwidth and the maximum frequency of part A, B, C (see text and (Apfelbeck et al. 2012) for further details).

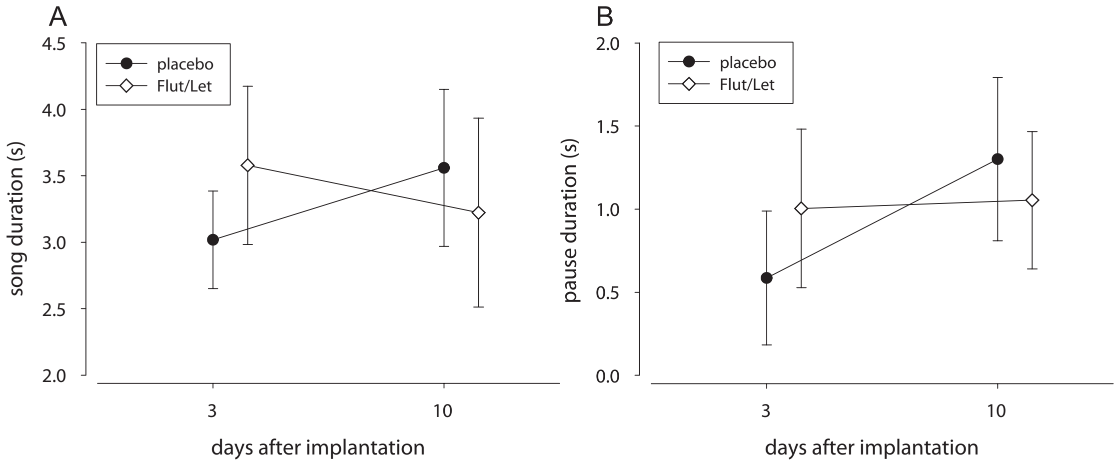

**Fig. 3.4 The duration of songs (A) and of pauses between part A and B (B).** Behaviors were shown in response to simulated territorial intrusions on day 3 and day 10 after treatment with a placebo or flutamide and letrozole (Flut/Let). Placebo-implanted males sang significantly longer songs with a longer pause between part A and B on day 10 than on day 3. Bars represent means and 95% CI.

**Table 3.2 Linear mixed model results for the effects of treatment (placebo- or Flut/Let-implanted) and day (3 or 10) after implantation on vocal behaviors**

| | **during STI** | | **after STI** | |
|---|---|---|---|---|
| **song duration** | | | | |
| treatment | $F_{1,16} = 0.07$ | p = 0.8 | $F_{1,18} = 2.8$ | p = 0.1 |
| day of STI | $F_{1,12} = 0.2$ | p = 0.7 | $F_{1,17} = 0.3$ | p = 0.6 |
| treatment*day | **$F_{1,12} = 8.4$** | **p = 0.01** | | |
| **duration pause A - B** | | | | |
| treatment | $F_{1,16} = 0.4$ | p = 0.6 | $F_{1,18} = 1.5$ | p = 0.2 |
| day of STI | **$F_{1,13} = 5.2$** | **p = 0.04** | **$F_{1,13} = 5.8$** | **p = 0.03** |
| treatment*day | $F_{1,13} = 3.8$ | p = 0.07 | | |
| **elements in A** | | | | |
| duration A | **$F_{1,12}=17.8$** | **p = 0.001** | $F_{1,12} = 0.2$ | p = 0.7 |
| treatment | $F_{1,16} = 0.1$ | p = 0.8 | $F_{1,18} = 0.2$ | p = 0.7 |
| day of STI | $F_{1,12} = 4.2$ | p = 0.06 | **$F_{1,12} = 6.7$** | **p = 0.02** |
| duration A*treatment | $F_{1,12} = 1.6$ | p = 0.2 | $F_{1,12} = 4.0$ | p = 0.07 |
| duration A*day | | | $F_{1,12}= 0.4$ | p = 0.5 |
| treatment*day | | | $F_{1,12} = 0.4$ | p = 0.5 |
| duration A*treatment*day | | | **$F_{1,12} = 4.7$** | **p = 0.05** |
| **frequency bandwidth A** | | | | |
| duration A | $F_{1,12} = 2.5$ | p = 0.1 | $F_{1,16} = 0.09$ | p = 0.8 |
| treatment | $F_{1,16} = 1.0$ | p = 0.3 | **$F_{1,18} = 5.2$** | **p = 0.04** |
| day of STI | $F_{1,12} = 0.004$ | p = 1.0 | **$F_{1,16} = 4.4$** | **p = 0.05** |
| duration A*treatment | $F_{1,12} = 3.4$ | p = 0.09 | | |
| **frequency bandwidth B** | | | | |
| duration B | $F_{1,6} = 0.07$ | p = 0.8 | $F_{1,15} = 3.1$ | 0.1 |
| treatment | $F_{1,15} = 0.02$ | p = 0.9 | **$F_{1,18} = 6.2$** | **p = 0.02** |
| day of STI | **$F_{1,6} = 13.7$** | **p = 0.01** | $F_{1,15} = 4.2$ | p = 0.06 |
| duration B*treatment | **$F_{1,6} = 56.1$** | **p = 0.0003** | | |
| duration B*day | **$F_{1,6} = 23.5$** | **p = 0.003** | | |
| treatment*day | **$F_{1,6} = 31.3$** | **p = 0.001** | | |
| duration B*treatment*day | $F_{1,6} = 4.6$ | p = 0.07 | | |
| **elements in C** | | | | |
| duration C | **$F_{1,11} = 13.7$** | **p = 0.004** | **$F_{1,15} = 8.1$** | **0.01** |
| treatment | $F_{1,16} = 0.3$ | p = 0.6 | $F_{1,18} = 0.005$ | p = 1.0 |
| day of STI | $F_{1,11} = 1.2$ | p = 0.3 | **$F_{1,15} = 6.3$** | **p = 0.02** |
| **frequency bandwidth C** | | | | |
| duration C | $F_{1,11} = 1.3$ | p = 0.3 | $F_{1,15} = 0.3$ | p = 0.6 |
| treatment | $F_{1,16} = 0.5$ | p = 0.5 | $F_{1,18} = 0.07$ | p = 0.8 |
| day of STI | $F_{1,11} = 0.01$ | p = 1.0 | $F_{1,15} = 0.8$ | p = 0.4 |

To control for repeated measures the ID of each territory owner was included as random intercept. Significant results are highlighted in bold.

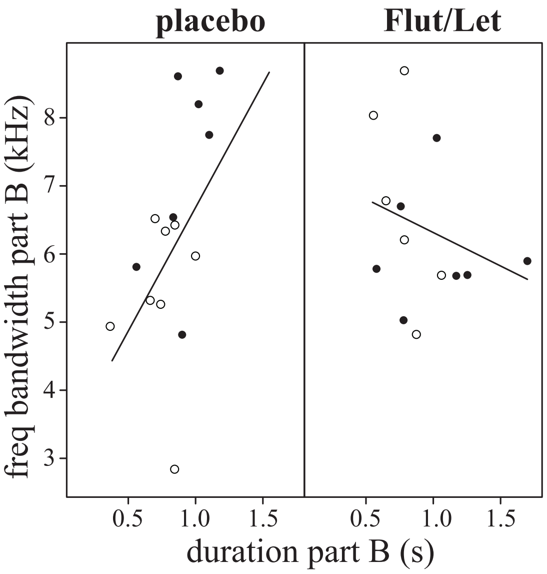

**Fig. 3.5 The frequency bandwidth (kHz) of part B plotted against the duration of part B of songs sang in response to the STI.** Data are shown separately for placebo- (left) and Flut/Let-treated males (right; filled circles: STI on day 3; open circles: STI on day 10). Lines represent regression lines for both days taken together.

## Non-vocal behavior after the STI

After the decoy was removed and the playback stopped, placebo- and Flut/Let-implanted males did not differ in the amount of time they spent within 10 m of the decoy (treatment: $F_{1,18} = 0.5$; day: $F_{1,18} = 1.7$, $p = 0.2$; interaction: $F_{1,18} = 0.2$, $p = 0.7$), in the number of head nods (treatment: $F_{1,18} = 0.2$, $p = 0.7$; day: $F_{1,17} = 0.1$, $p = 0.7$; interaction: $F_{1,17} = 0.1$, $p = 0.8$) or in the number of songs (treatment: $F_{1,18} = 0.004$, $p = 1.0$; day: $F_{1,18} = 3.1$, $p = 0.1$; interaction: $F_{1,18} = 0.4$, $p = 0.5$).

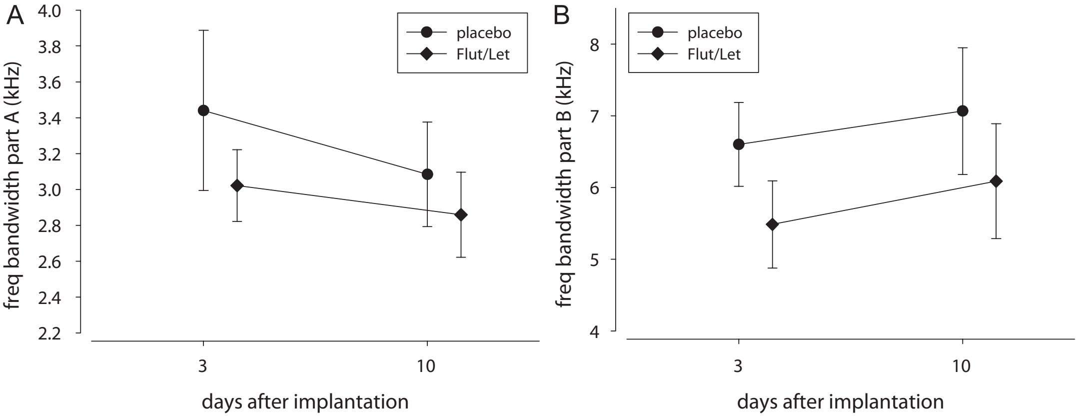

**Fig. 3.6 Frequency bandwidth (kHz) of song parts A (A) and B (B).** The song was recorded after simulated territorial intrusions on day 3 and day 10 after treatment with a placebo or flutamide and letrozole (Flut/Let). Placebo-implanted males sang both parts with a significantly broader frequency bandwidth than Flut/Let-implanted males. For details on song measurements and statistics see text.

## Vocal behavior after the STI

After the STI, placebo- and Flut/Let-implanted males differed significantly in song structure: Flut/Let-implanted males sang parts A and B after both STIs with a smaller frequency bandwidth than placebo-implanted males (Table 3.2, Fig. 3.6).

Furthermore, males of both treatment groups changed structural features of their song from day 3 to day 10: they sang with longer pauses between parts A and B on day 10 compared to day 3 (Table 3.2) and part C with more elements on day 10 than on day 3 (Table3.2). In addition, when controlling for the duration of part A, placebo-implanted males sang more elements in this part on day 10 than on day 3 (Table 3.2, Fig. 3.7). Also, Flut/Let-implanted males tended to repeat more elements in part A on day 10 than on day 3, but they did so with a shorter duration of part A compared to control males (Table 3.2, Fig. 3.7).

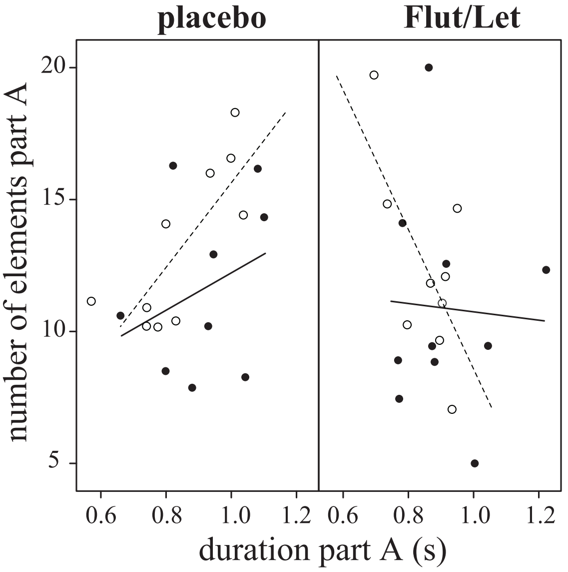

**Fig. 3.7 The number of elements in part A plotted against the duration of the part A of songs sang after the STI.** Data are presented separately for placebo- (left) and Flut/Let-treated males (right; filled circles and solid lines: STI on day 3; open circles and dashed lines: STI on day 10). Lines represent regression lines.

Thus, song sung by Flut/Let-implanted males after the STI was in general of potentially lower competitive value than that of placebo-implanted males (smaller frequency bandwidth of part A and B). In addition, after the STI males of both treatment groups tended to sing song that was probably of higher competitive value on day 10 than on day 3 (more elements in part A and C).

## Discussion

In a breeding context inhibiting the effects of testosterone and estrogen did not prevent male black redstarts from successfully defending their territories and had no direct effect on the non-vocal territorial response or song output. However, after the STI the song structure of Flut/Let-implanted males differed significantly from that of placebo-implanted males, especially in those parts of their song that males enhance in response to a territorial intruder (Apfelbeck et al. 2012). Furthermore, we found a surprising effect of day of STI. We had challenged males 3 and 10 days after implantation, because in some studies the effect of pharmacologically inhibiting the action of androgens was only apparent after several days to weeks (Schwabl and Kriner 1991; Soma et al. 1999). In line with these studies, the non-vocal territorial response of Flut/Let-implanted males was reduced during the second STI 10 days after implantation compared to the STI only 3 days after implantation. However, when compared directly, the non-vocal territorial response did not differ between placebo- and Flut/Let-treated males, neither on day 3 nor on day 10. Furthermore, in contrast to the expectation that day of STI may have had an effect in Flut/Let-implanted males, it also had an effect in placebo-implanted males: placebo- and Flut/Let-implanted males reduced their vocal response to the STI from day 3 to day 10, but the effect was stronger in placebo- compared to Flut/Let-implanted males. After the respective STIs, males of both groups enhanced their vocal response from day 3 to day 10 (but Flut/Let males less so than placebo-implanted males). Thus, in both treatment groups there was a significant effect of day of STI, which may reflect an effect of experience. We have shown previously in black redstarts, that challenging males with a STI on three consecutive days significantly changed their behavior towards a territorial intruder: they approached the intruder faster and tended to spent more time close to the decoy on day 3 than on day 1 (Apfelbeck et al. 2011). The present experiment suggests that there is still an effect of experience even with a gap of 7 days between the experiments and that the treatment significantly influenced this effect. The data suggest that both treatment groups changed the emphasis of the territorial response from during to after the STI from day 3 to day 10. Furthermore, blocking androgen and estrogen action changed the emphasis of the territorial response during day 3. Placebo-implanted males showed a stronger vocal response (as evident in song structural changes from day 3 to day 10), while experimental males responded more with direct approach and non-vocal threat behaviors (as evident in changes in these behaviors from day 3 to day 10; summarized in Table 3.3).

**Table 3.3 Relative change in behavior from day 3 to day 10 in response to the STI.**

| | | placebo | Flut/Let |
|---|---|---|---|
| **non-vocal response** | time within 5 m | | ↓ |
| | feather fluffing | | ↓ |
| **vocal response** | number of songs | ↑ | ↑ |
| | song duration | ↑ | |
| | pause A - B | ↑ | |
| | freq bandwidth B<br>(duration part B) | ↓ | |
| | elements in A | ↓ | ↓ |

Downward arrows (↓) indicate a quantitative decrease of the behavior from day 3 to day 10; upward arrows (↑) indicate a quantitative increase of the behavior from day 3 to day 10. The emerging pattern suggests that both treatment groups reduced their territorial response during the STI from day 3 to day 10, but that this change mainly concerned non-vocal behaviors in blocker-implanted males, but vocal behaviors in placebo-implanted males.

Thus, black redstarts reacted similarly to most other songbird species, in which treatment with androgen inhibitors during the breeding life history stage did not reduce overall territorial behavior, but only some components of it (Table 3.1, but see (Soma et al. 1999)) and supports the view that testosterone emphasizes vocalizations (structure and/or output) within the territorial response. For example, male red-winged blackbirds, *Agelaius phoeniceus,* implanted with an androgen receptor blocker and an aromatase inhibitor spent more time on their territories engaging in aggressive interactions and vocalizations compared to control males, but still lost parts of their territories (Table 3.1; (Beletsky et al. 2010)). It is unknown why male red-winged blackbirds implanted with anti-androgens and an aromatase inhibitor were less able to defend their territories, but possibly the treatment could have had an effect on song structure and thereby the quality of the song. This remains speculative, however, because song structure was not measured in the study on red-winged blackbirds. Male spotted antbirds, *Hylophylax n. naevioides*, implanted with anti-androgens and aromatase inhibitors do not sing at all and produce fewer aggressive calls in response to a staged male-male encounter in captivity compared to control males while non-vocal behaviours were not influenced by the treatment (Hau et al. 2000). Male European robins, *Erithacus rubecula,* approached intruders more conspicuously by singing from perches above the intruder during the breeding season than during the acquisition of non-breeding territories, even though the quantitative response (latency to approach, time spent close to the intruder, song output) did not differ between seasons (Schwabl and Kriner 1991). Also in this study song structure was not assessed, but the authors suggested that sexually selected components of the song may be under androgenic influence. Our results strengthen this idea: in species in which territorial behavior is not restricted to the breeding life history stage, testosterone and estradiol may facilitate only specific components of territorial behavior that are important in a reproductive, i.e. mating and breeding, context. The specific components that are altered by steroids may, for example, act as signals for females. Song during territorial contests in the breeding season is not only directed towards intruding or neighboring males, but may also convey information to the mate or other females (Hau et al. 2000). Females pay attention to the performance of their mates during territorial challenges, which may influence female behavior, i.e. the decision whether they engage in extra-pair copulations or not (Hau et al. 2000). These features may differ between species (e.g. song rate or song structure), may signal male quality and can be correlated with variation in testosterone levels (Galeotti et al. 1997). In other studies on black redstarts we have shown that males are less likely to respond to a simulated territorial intrusion with song during non-breeding territoriality in autumn (when testosterone levels are low) than during breeding territoriality in spring, while the non-vocal territorial response was not reduced during non-breeding compared to breeding (Apfelbeck and Goymann 2011). Furthermore, in male black redstarts the structural changes in song in response to territorial intrusions seem to depend partly on testosterone and/or estradiol, because both males that were implanted with an anti-androgen and an aromatase inhibitor in a breeding context and males that were challenged in a non-breeding context when testosterone levels were naturally low, did not show the full structural change of their song (Apfelbeck et al. 2012). So far, we are only aware of one further study that incorporated measures of song structure when testing the role of androgens and estrogens in the regulation of territoriality (see Table 3.1). This study on great tits, *Parus major*, did not find an effect of anti-androgens and aromatase inhibition on song structure (Van Duyse et al. 2005). However, the great tit study assessed effects of the treatment on spontaneously produced dawn song and not song in response to a simulated territorial intruder as in our study. Nevertheless, our data suggest that in male black redstarts and potentially other species as well testosterone and/or estrogens may shift the focus of the territorial response to vocal behaviors and facilitate structural changes in the song within an agonistic context during the breeding life history stage.

***Dependence of territoriality on androgens and androgen responsiveness to male-male interactions.***

Some bird species show an increase in testosterone after male-male interactions during breeding, whereas others do not (reviewed in (Goymann et al. 2007a; Goymann 2009)). These short-term increases of testosterone are thought to enhance the persistence of the territorial response (Wingfield 1994a), may induce the winner effect (Oyegbile and Marler 2005; Oliveira et al. 2009) and are a phenomenon found across all vertebrate classes (Wingfield et al. 1990; Hirschenhauser et al. 2003; Archer 2006; Hirschenhauser and Oliveira 2006). Surprisingly, though, these surges in testosterone are absent in many bird species (Goymann et al. 2007a; Goymann 2009). When territorial behavior as such is decoupled from the control of testosterone in a reproductive context, as for example in the black redstart, territorial disputes may also not induce short-term increases in testosterone. We have previously shown that male black redstarts do not increase testosterone during simulated and real territorial encounters with other males (Apfelbeck and Goymann 2011), but obviously this does not prevent them from enhancing their territorial response during future territorial encounters (Apfelbeck et al. 2011). Song sparrows, in contrast, increase testosterone during simulated territorial intrusions (Wingfield and Wada 1989) and this increase seems to enhance the persistence of the territorial response after the stimulus is withdrawn (Wingfield 1994b). Furthermore, inhibition of aromatase reduces the whole suite of territorial behaviors in response to an intruder in song sparrows, even though the effect is less obvious during the breeding than during the non-breeding season (Soma et al. 2000a). Why species differ in the hormonal control of aggression displayed in different life history contexts and short-term territorial aggression is still unclear (Wingfield and Hunt 2002; Goymann et al. 2007a; Lynn 2008; Goymann 2009; Apfelbeck and Goymann 2011) and highlights a potential diversity of physiological mechanisms that is largely unexplored (Spicer and Gaston 1999).

### Conclusions

In some species that defend a territory during and outside a breeding life-history context, territorial behavior even during breeding may be decoupled from testosterone or its metabolites. Rather, testosterone or estradiol may change particular components of the territorial repertoire that are specifically relevant in a breeding context. Testosterone may shift the emphasis of the territorial response to these components. Thereby, these behaviors may change in signal value and may in turn indicate male quality to other males (intruders and neighbors), but also to females witnessing the territorial dispute. Such context-dependent changes in song structure during the breeding life history stage may be facilitated by seasonal changes in testosterone levels. There seem to be fundamental differences between species to which degree territorial behavior is regulated by testosterone or its metabolites in different life history contexts. These differences may be directly related to variation in androgen responsiveness to male-male interactions, which in turn may depend on life-history and ecological characteristics of a particular species.

## Material and Methods

### Capture and implantation with androgen receptor blockers and aromatase inhibitors

Adult (≥ 2 years) male black redstarts were caught in 2009 between April 9th and 27th in Upper Bavaria (N 47°, E 11°, 500–600 m above sea level) with mealworm-baited ground traps. Birds were lured to the traps by broadcasting playbacks with the species' song of short duration (< 2 min). We remotely muted the loudspeaker as soon as the territory owner approached the traps. Conspecific playback does not influence testosterone levels in territorial male black redstarts (Apfelbeck and Goymann 2011; Apfelbeck et al. 2011). Upon capture we measured the birds and implanted males with either one placebo pellet (n = 10) or two time release pellets (n = 10) containing the androgen receptor blocker flutamide and the aromatase inhibitor letrozole, respectively (21 day release: 1.5 mg per pellet; release rate 71 µg/day; Ø = 3.2mm, Innovative Research of America, Sarasota, FL). Letrozole inhibits cytochrome p450 aromatase (CYP 19). This enzyme is important for the conversion of testosterone to estrogen (Cheshenko et al. 2008). Thus, by combining flutamide and letrozole, it is possible to block direct and indirect effects of androgens on behavior (Schlinger and Callard 1990; Balthazart 1997). Implants were inserted subcutaneously with a pair of tweezers through a small incision in the skin on the back between the wings. The incision was sealed with tissue glue (Nexaband; World Precision Instruments).

Control and experimental groups did not differ significantly in body mass (t = 1.52, df = 17.9, p = 0.15), length of the right tarsus (t = -0.25, df = 12.5, p = 0.8), length of the right wing (t = 0.25, df = 14.2, p = 0.8) and cloacal protuberance (CP) volume (t = -0.17, df = 13.1, p = 0.9), which was estimated by calculating the volume of a cylinder ($V = \pi*(\text{CP width}/2)^{2}*\text{CP height}$). Each male was banded with a numbered aluminium ring (Vogelwarte Radolfzell) and a unique combination of three color rings for individual recognition. Measuring, ringing and implanting the birds took no longer than 25 min after which the males were released onto their territories. All experimental procedures were approved by the Committee on the Ethics of Animal Experiments of the governmental authorities of Upper Bavaria (Permit Number: Az. 55.2-1-54-2531-151-08).

## Effectiveness of androgen receptor blockers and aromatase inhibitors

To assess if the treatment with the androgen receptor blocker and the aromatase inhibitor was effective we caught another set of males with the same method as described above in April 2009 and 2010 and in September 2010, took a blood sample immediately upon capture (within 5 min) and brought them to the laboratory. In 2009 five males were caught, implanted with flutamide and letrozole and bled a second time 3 days after implantation. In 2010 we caught another 16 males (8 in April and 8 in September) that were bled upon capture, were then either implanted with flutamide and letrozole or with flutamide only, and bled again 3 and 10 days after implantation. In all cases pellets were still visible when we took the blood samples. Males were held in individual cages under simulated natural photoperiod and released onto their respective territories after taking the last blood sample. Testosterone concentration was determined by direct radioimmunoassay following the procedure described in (Goymann et al. 2006; Apfelbeck and Goymann 2011). Mean ± SD efficiency of the extraction with dichloromethane was 93 ± 3% for the samples collected in 2009 and 85 ± 5% for those collected in 2010. Samples were measured in duplicates and in separate assays (2009 + 2010). The lower limit of detection of the assay was determined as the first value outside the 95% confidence intervals for the zero standard ($B_{max}$) and was 2.6 (2009), 4.5 (2010) pg/ml. The intra-assay coefficients of variation were 1.2% (2009) and 2.9% (2010), respectively. The inter-assay variation was 3.2%.

The combined implantation of flutamide and letrozole significantly increased plasma testosterone levels within 3 days as compared to levels before implantation (paired t-test, n = 9, t = -3.4, p = 0.01, Table 3.4) and testosterone levels were still significantly elevated after 10 days (Wilcoxon test, U = 0, p = 0.003, Table 3.4). These data suggest that letrozole effectively inhibited the aromatization of testosterone to estrogen, because in birds the negative feedback regulation of testosterone is achieved via estradiol, which inhibits gonadotropin secretion (Bhatnagar et al. 1992; Zumpe and Michael 1994; Soma et al. 1999). Thus, the increase in testosterone indicates that there is no estradiol that would be capable to induce this negative feedback. As soon as estradiol levels would rise, negative feedback would kick in and as a consequence reduce circulating testosterone levels. This happens when flutamide is implanted without letrozole during the breeding season (Table 3.4). Hence, elevated testosterone levels provide evidence that letrozole blockage works effectively during breeding (see also (Soma et al. 1999; Fusani et al. 2001; Fusani and Hutchison 2003; Hau et al. 2004; Hau and Beebe 2011)). During non-breeding, when the testes are regressed, flutamide and letrozole treatment does not result in an increase in testosterone (Friedman chi-squared = 0.25, df = 2, p = 0.9, Table 3.4).

**Table 3.4 Plasma testosterone levels (ng/ml ± 95% CI) before implantation, and 3 and 10 days after implantation of either flutamide alone or flutamide and letrozole combined.**

| testosterone (ng/ml) | before | 3 days after | 10 days after |
|---|---|---|---|
| **breeding** | | | |
| flutamide/letrozole | 1.02 ± 0.4 (n= 9) | 5.02 ± 2.0 (n= 9) | 5.9 ± 2.1 (n= 9) |
| flutamide | 2.2 ± 6.6 (n= 4) | 2.2 ± 3.4 (n= 4) | 1.2 ± 1.5 (n= 4) |
| **non-breeding** | | | |
| flutamide/letrozole | 0.06 (n= 4) | 0.04 (n= 4) | 0.04 (n= 4) |
| flutamide | 0.04 (n= 4) | 0.05 (n= 4) | 0.08 (n= 4) |

As flutamide is a competitive inhibitor of androgen receptors (Neumann et al. 1977) the increase in testosterone levels caused by letrozole could theoretically have compromised the effectiveness of flutamide. However, this is very unlikely, as the dosage used (1.5 mg flutamide and a release rate of 71 µg/day) resulted in a concentration of flutamide that is roughly 700 times higher than the maximum concentration of testosterone measured in a black redstart. This surplus of flutamide should have been sufficient to effectively inhibit androgen action.

## Playback stimuli

Songs used as playbacks were recorded in spring 2009 with a Sennheiser directional microphone (ME66/K6) connected to a Marantz solid state recorder PMD 660 (sampling frequency: 44.1 kHz; resolution: 16 bit) from 20 different males that were at least 10 km away from our focal males. Playbacks were created using Avisoft Saslab pro software version 4.51 (Raimund Specht, Berlin, Germany, for details see (Apfelbeck et al. 2012)). Each playback consisted of 20 songs recorded from one male. Each playback was used only once in each experimental group, but the same playbacks were randomly used during the STIs on day 3 and 10. Thus, each male was challenged with two different playbacks.

## Simulated territorial intrusion

To assess the effect of the Flut/Let treatment on territorial behavior we performed STI experiments three and ten days after implantation by placing a stuffed decoy into the center of the territory of a focal male and playing back black redstart song as described above. As decoys we used three different stuffed males in full adult plumage that were protected by an inconspicuous cage made of a wire frame and mist net material and mounted on a tripod. A string attached to the wire frame allowed us to remotely remove the decoy by pulling the string from a distance of about 30 m into a plastic cylinder below the wire frame. We put a remote-controlled loudspeaker (Foxpro Scorpion, digital game caller, FOXPRO Inc. Lewistown, USA) underneath the decoy to play back the territorial song of a potential rival at a sound pressure level of 65 dB SPL at 1 m (as measured with a CEL 573.B1 Sound Level Analyzer). The behavioral response of male black redstarts to simulated territorial intrusions varies from moving to an exposed singing post and increasing the song output to approaching the decoy and threat posturing, which – in some cases – may cumulate into an attack (Landmann and Kollinsky 1995a; Van Duyse et al. 2002). Therefore, we recorded the following behaviors of the territory owner during the STI for 20 min: (1) latency to respond to the STI either by singing or approaching the decoy, (2) the first time the male entered the area of 5 m around the decoy, (3) the time the male spent within 5 m of the decoy, (4) the time the territory owner spent with its feathers fluffed, (5) the number of head nods and (6) the number of flights over the decoy. The latter two behaviors are typical threat postures of male black redstarts (Landmann and Kollinsky 1995a). Furthermore, we noted whenever the male attacked the decoy. During the whole time we also recorded the song of the territory owner using a Sennheiser directional microphone (ME66/K6) connected to a Marantz solid state recorder PMD 660. Usually we could determine the location of the male during the whole STI, however, sometimes it was hidden from view and we could not correctly record head nods and fluffing behavior. Therefore, we also noted when we knew the location of the bird but could not see it. After 20 min the playback was remotely muted and the decoy removed and the behavior of the territory owner observed for another 10 min. After the STI we recorded the time the territory owner spent within 10 m of the decoy instead of 5 m. Behavioral observations were conducted blind to the treatment of the focal males and always by the same observer from at least 30 m distance. Song recordings were made by a second observer.

## Song analysis

Song was analyzed using again Avisoft-SASLab Pro software, version 4.51. Recordings were visualized in spectrograms (settings: sample rate 22,050 Hz, FFT = 256 points, Hamming-Window, Overlap: 50%). We determined the number of songs by visual inspection and selected songs solely based on their recording quality (low background noise) for further sound analysis. Each song of black redstarts can be divided into three distinct parts (part A, B and C, see (Cucco and Malacarne 1999; Apfelbeck et al. 2012) and Fig. 3.3 for more details) with a pause of varying duration between part A and B. We measured the duration of parts A, B, C, the total song and the duration of pauses between A and B. We counted the number of elements of part A and C (mean of max. 20 songs). We also determined the frequency bandwidth and the maximum frequency of part A, B and C using the automatic parameter measurement function (threshold -20 dB) in Avisoft (mean of max. 10 renditions of high-quality songs).

## Statistical analysis

Data analysis was done with R version 2.9.1 (R Core Team 2014). Behavioral data and measures of song structure were analyzed with linear mixed models for the effects of treatment and day after implantation. We analyzed the behavior during and after the STI separately. To control for repeated measures we included bird ID as a random effect. After the STI we compared the time spent within 10 m of the decoy instead of 5 m as most males left the immediate surroundings of the decoy and went to higher singing posts. Also, because most males stopped feather fluffing after the decoy had been removed, we only analyzed the number of head nods after the STI. When analyzing the time within 5 m during the STI we included response latency as covariate in the models. For the analysis of treatment effects on the number of head nods and time spent feather fluffing we controlled for differences in the total amount of time we actually saw the bird. For treatment effects on song structural parameters we included the average duration of a song part as covariate in our models. Based on previous findings we assume that structural changes in the song in response to a simulated territorial intruder result in song of higher competitive value than song produced spontaneously (Apfelbeck et al. 2012).

In all cases we started with full models and removed interactions if they were above $\alpha > 0.1$. Experimental factors were always retained in the models. Dependent variables were transformed if assumptions of normality and/or equality of variances were not met. Significance was accepted at $\alpha \leq 0.05$. Sample sizes may deviate from 20 males in total as not all males sang during the STI and depending on the quality of the recording.

# Acknowledgements

We thank Novartis for kindly providing us with letrozole. We also want to thank Manfred Gahr and the Max-Planck Gesellschaft for funding this study, Alexander Weber, Maria Apfelbeck, and Matthias Schneider for help in the field, Sue Anne Zollinger, Henrik Brumm, Manfred Gahr and Gabriel Beckers for stimulating discussions and Michaela Hau for helpful comments on previous versions of the manuscript. We thank two referees, who provided helpful and constructive criticism to further improve the manuscripts. The authors declare no conflict of interest.

# CHAPTER 4 Testosterone affects song modulation during simulated territorial intrusions in male Black redstarts (*Phoenicurus ochruros*)

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# Abstract

Although it has been suggested that testosterone plays an important role in resource allocation for competitive behavior, details of the interplay between testosterone, territorial aggression and signal plasticity are largely unknown. Therefore, we investigated if testosterone acts specifically on signals that communicate the motivation or ability of individuals to engage in competitive situations in a natural context. We studied the black redstart, a territorial songbird species, during two different life-cycle stages, the early breeding phase in spring and the non-breeding phase in fall. Male territory holders were implanted with the androgen receptor blocker flutamide (Flut) and the aromatase inhibitor letrozole (Let) to inhibit the action of testosterone and its estrogenic metabolites. Controls received a placebo treatment. Three days after implantation birds were challenged with a simulated territorial intrusion (STI). Song was recorded before, during and after the challenge. In spring, both treatment groups increased the number of elements sung in parts of their song in response to the STI. However, Flut/Let-implanted males reacted to the STI with a decreased maximum acoustic frequency of one song part, while placebo-implanted males did not. Instead, placebo-implanted males sang the atonal part of their song with a broader frequency range. Furthermore, placebo-, but not Flut/Let-implanted males, sang shorter songs with shorter pauses between parts in the STIs. During simulated intrusions in fall, when testosterone levels are naturally low in this species, males of both treatment groups sang similar to Flut/Let-implanted males during breeding. The results suggest that song sung during a territorial encounter is of higher competitive value than song sung in an undisturbed situation and may, therefore, convey information about the motivation or quality of the territory holder. We conclude that testosterone facilitates context-dependent changes in song structures that may be honest signals of male quality in black redstarts.

# Introduction

Sexually selected signals often serve both to attract a mate and to advertise competitive abilities, for example during territorial disputes (reviewed in (Searcy and Nowicki 2005)). Studying the song of male passerine birds may advance our understanding of the mechanisms underlying the use and coordination of such signals. A range of studies revealed that song characteristics can transfer information about the quality of the singer (reviewed in (Gil and Gahr 2002)) such as its immune system (Garamszegi et al. 2004), age (reviewed in (Kipper and Kiefer 2010)), early experience (Searcy et al. 2010) or motivation to contribute to breeding (Borowiec and Halupka 2006).

In many species males modulate their song in an aggressive context: they might select certain song types matching a rival (Searcy and Beecher 2009), or produce specific song elements only in situations of high arousal (Bartsch et al. 2012). In addition, birds can change song characteristics such as frequency patterns and trill rate (Price et al. 2006; DuBois et al. 2009). Male as well as female listeners respond differentiated to such modulations (Drăgănoiu et al. 2002, 2006; Illes et al. 2006; DuBois et al. 2011). Song modulations can occur on two domains: on the one hand, birds may change the general output of song (e.g. song rate or amplitude), i.e. measures that potentially every male can vary within broad limits. On the other hand, modulation also occurs in structural song characteristics. Structural characteristics describe, for example, song repertoire characteristics (Voigt and Leitner 2008) or song parts that are challenging to sing, such as rapid broadband trills (reviewed in (Podos et al. 2009)), specific song trills (Vallet et al. 1998) or consistent syllables (Botero et al. 2009). Structural song patterns have been classified as 'index signals' that honestly communicate a physical trait related to male quality (Hurd and Enquist 2005). Only very few studies have revealed a capability of individuals to modulate such physically constrained signals within narrow limits (Bee and Perrill 1996; Price et al. 2006; DuBois et al. 2009; Weiss et al. 2012). Thus, from a functional point of view, index signals such as structural song parameters should play an important role in the communication of competitive ability.

The steroid hormone testosterone plays an important role in the regulation of adult singing and territorial behaviors and the associated vocalizations during breeding are facilitated by testosterone in a wide range of male vertebrates (reviewed in (Newman 2007), (Harding 2004). Therefore, it has been suggested that testosterone might play an important role in resource allocation for competitive behavior during reproduction (reviewed in (Wingfield et al. 2006)). From this point of view, testosterone should act specifically on signals that communicate the motivation or ability of individuals to engage in competitive situations and is, therefore, expected to be involved in context-dependent adjustment of such signals. However, details of the interplay between hormones, territorial aggression and signal plasticity in a natural context are largely unknown.

Manipulations of testosterone levels may alter song output (measured, for example, as song rate or duration; e.g. (Silverin 1980; Nolan et al. 1992; Hau et al. 2000; Boseret et al. 2006; Ritschard et al. 2011)). Whether testosterone also affects structural song parameters is less clear. In barn swallows (*Hirundo rustica*), the duration and pulse rate of the harsh 'rattle' element correlated moderately with absolute testosterone levels (Galeotti et al. 1997). Manipulation studies suggested that zebra finches (*Taeniopygia guttata*) treated with testosterone decreased the fundamental frequency of harmonic stacks in their song (Cynx et al. 2005). Other correlational and experimental studies with testosterone treatment failed to find effects on structural song parameters (Weatherhead et al. 1993; Kunc et al.

2006; Ritschard et al. 2011). Studies that implant birds with testosterone may be problematic, because especially immediately after implantation testosterone may circulate in pharmacological doses (Fusani 2008; Edler et al. 2011). It is thus questionable whether manipulations exclusively within the physiological range of testosterone would reveal similar results. Treatments inhibiting the action of testosterone or its major metabolite estradiol by blocking the androgen receptor and/or the conversion to estradiol avoid such pharmacological effects (but can only inhibit, not enhance effects of steroid hormones). The – so far - only study in which the androgenic and estrogenic pathways of testosterone action were blocked failed to find effects on structural song parameters in great tits (*Parus major*, (Van Duyse et al. 2005)). Thus, to the best of our knowledge, an effect of physiological changes in testosterone on structural measures of song has not been demonstrated so far.

Song plasticity and its potentially underlying hormonal mechanisms may be studied in different contexts, such as during spontaneous singing or singing during a territorial challenge because the song used (output and structure), the information transmitted (e.g. quality and/or competitive ability) and the receiver and/or audience (other males and/or females) may differ in these contexts. Thus, depending on context, song may be facilitated by sex steroids or not. Furthermore, several songbird species also sing outside the breeding season, providing an additional contextual variable. Song characteristics (of spontaneous song) differ between the breeding – and non-breeding season: For example, some species produce more repetitive elements (Dewolfe et al. 1974; Smith et al. 1997; Leitner et al. 2001; Voigt and Leitner 2008), longer songs (Riters et al. 2000), and more stereotypic song (Smith et al. 1997) in spring than in fall. Comparisons between breeding and non-breeding song were so far restricted to spontaneously produced song. Whether changes in song during a territorial challenge also differ between seasons has not been studied yet. Such a difference should be expected from a functional point of view, since fall territories are not of immediate importance for reproduction. Those seasonal differences in song might well be mediated by testosterone levels, because in most songbird species testosterone levels are low during non-breeding (Logan and Wingfield 1990; Schwabl 1992; Wingfield 1994a; Landys et al. 2010). It remains open, however, whether and how testosterone is involved in context-dependent song plasticity during the non-breeding season (e.g. (Logan and Fulk 1984)). In song sparrows, for example, testosterone also regulates territorial behavior during the non-breeding season, presumably through steroids of non-gonadal origin that are then metabolized to testosterone and estradiol directly in the brain (Schlinger et al. 2001).

In this study, we investigated the role of testosterone in regulating spontaneous song and song in an aggressive context in free-living male black redstarts (*Phoenicurus ochruros*). The species is well-suited to study this topic as there is evidence that song structures may contain information about the competitive ability or motivation (Cucco and Malacarne 1999). Black redstarts show delayed song maturation, i.e. adult and yearling males differ in structural song parameters (Cucco and Malacarne 1999) such as the duration of song parts and number of elements or frequential song patterns, as well as in visual traits (delayed plumage maturation) (Landmann and Kollinsky 1995b). These age-differences are also reflected in simulated territorial intrusions: adult and yearling males respond differentially to playbacks of the two age classes (Cucco and Malacarne 1999). Despite this delayed maturation, adults as well as one year olds reproduce, but adult males usually occupy higher quality habitats and have a higher reproductive success (Landmann 1996). Although this has not yet been studied, it seems plausible to assume that behavioral and morphological age-differences may be accompanied by different hormonal profiles.

Furthermore, black redstarts not only defend territories in spring after having returned from their wintering grounds, but also in fall, just after molt and before migration (Weggler 2000). During the territorial phase in fall they have low testosterone levels (Apfelbeck and Goymann 2011).
Against this background, we tested the role of testosterone in the modulation of song characteristics in this species. We did so by implanting birds with an androgen receptor blocker (flutamide) and an aromatase inhibitor (letrozole) which inhibits the conversion of testosterone to estradiol, as testosterone can modulate behavior either directly by binding to androgen receptors or indirectly by conversion to estradiol and binding to estrogen receptors (Balthazart 1997). As controls we used birds treated with placebo implants. After implantation, we first recorded the spontaneous song of territorial males in an undisturbed context and then conducted a playback experiment simulating a territorial intrusion (STI) by a foreign male. This procedure was conducted in spring during the early breeding season, and again in fall during non-breeding, using a different set of birds.

The aim of our study was threefold. First, we wanted to investigate whether black redstarts change structural song parameters in an aggressive context, i.e. whether song parameters differ between a non-challenged context before the STI and during/after the STI. Based on prior studies on black redstart song and in particular on a playback-study on song and age ((Cucco and Malacarne 1999), see above) we expected to find changes in song output measures and structural song characteristics. Index signals that honestly communicate a physical trait related to male quality (Hurd and Enquist 2005) are good candidates here. Thus, we expected those structural song parameters to change in the agonistic context that have been shown to be characteristic for adult males song, that is the number of song elements and the frequency-range of song parts (Cucco and Malacarne 1999). Specifically we would expect focal males to sing longer song parts with trills, higher frequencies and/or with broader frequency bandwidth during a territorial encounter than in an undisturbed situation.

Second, by blocking the actions of testosterone, we attempted to determine the role of this hormone in context-dependent vocal plasticity. If testosterone is playing a key role in the resource allocation for competitive behavior (e.g. (Hau 2007)) during the breeding season in spring, we would expect flutamide/letrozole-treated males (thereafter termed Flut/Let males) to invest less in those behaviors and song patterns that are relevant in such situations than placebo-males. Thus, changes in song during territorial encounters (see above) should be less pronounced or absent in Flut/Let males in contrast to placebo treated males.

Thirdly, because testosterone levels are low in fall (Apfelbeck and Goymann 2011) males should not change song parameters in an aggressive context in fall and the treatment with flutamide and letrozole should have no effect on song. We therefore, compared song behavior in an undisturbed and an agonistic context in fall again between Flut/Let males and placebo males. We predicted that in fall Flut/Let- and placebo-implanted males should not differ in their vocal response to a simulated territorial intrusion.

## Materials and Methods

### Ethics Statement

All experimental procedures were approved by the Committee on the Ethics of Animal Experiments of the governmental authorities of Upper Bavaria (Permit Number: Az. 55.2-1-54-2531-151-08). All surgery was performed under local carprofen anesthesia, and all efforts were made to minimize suffering.

### Study Site and Subjects

Adult territorial male black redstarts were caught during spring (9–27 April 2009) and fall (22 September - 7 October 2009) in Upper Bavaria, Germany (N 47°, E 11°) with mealworm-baited ground traps. To avoid potential age-related differences in song and hormonal profile (see Introduction) we restricted our study to males being 2 yrs or older. Birds were lured to the traps by broadcasting playbacks of the species' song of short duration (<2 min). We remotely muted the loudspeaker as soon as the territory owner approached the traps. Conspecific playback does not influence testosterone levels in territorial male black redstarts (Apfelbeck and Goymann 2011). Males were implanted with either one placebo pellet (spring: n=10, fall: n=6) or two time release pellets (spring: n=10, fall: n=6, 21 day release: 1.5 mg per pellet; release rate 71 µg/day; Ø=3.2 mm, Innovative Research of America, Sarasota, FL) containing the androgen receptor blocker flutamide (Sigma-Aldrich F-9397) and the aromatase inhibitor letrozole (Novartis; (Cheshenko et al. 2008)), respectively. Each male was implanted only once, either in spring or fall. Implants were inserted subcutaneously on the back between the wings through a small incision in the skin that was sealed with tissue glue afterwards (Nexaband; World Precision Instruments).

Control and experimental groups did not differ significantly in body mass (spring: t=1.52, df=17.9, p=0.15, fall: W=34.5, n=16, p=0.8), length of the right tarsus (spring: t=−0.25, df=12.5, p=0.8, fall: W=44, n=16, p-value=0.2), length of the right wing (t=0.25, df=14.2, p=0.8, fall: W=41, n=16, p-value=0.4) and cloacal protuberance (CP) volume (spring: t=−0.17, df=13.1, p=0.9), which was estimated by calculating the volume of a cylinder [V=π*(CP width/2)2*CP height].

Each male was banded with a numbered aluminum ring (Vogelwarte Radolfzell) and a unique combination of three color rings for individual recognition. Measuring, ringing and implanting the birds took no longer than 25 min after which the males were released onto their territories.

## Experimental Design

Simulated territorial intrusions were conducted three days after implantation. All experiments were conducted between 8：00 and 12：00 h. For the playback we used spontaneous songs from 20 adult male black redstarts recorded in spring 2009 and from 12 adult males recorded in fall 2009 with the same equipment as reported below. For each target male we selected a playback that was recorded at least 10 km away from the study area. This distance was sufficient to avoid that target birds might know the stimulus birds. Playbacks were put together using Avisoft-SASLab Pro software, version 4.51 (Raimund Specht, Berlin, Germany). Each playback consisted of 20 songs recorded from one male. Songs were filtered (1 kHz high-pass filter) and amplitude was normalized to 75%. A playback consisted of each of two different song types (X and Y) played back in a XXYYXXYYXX sequence, with X and Y in 10 different versions (i.e. different exemplars of the same type). Songs were divided by pauses of 4.5 s. By repeating each sequence six times the playback had a duration of 20 minutes in total. This design resembled the organization of natural song in this species.

We presented each of the playbacks twice: once to a Flut/Let bird and once to a control bird, thereby alternating the presentation order between the two groups. These pairs of groups were tested in close temporal proximity in order to rule out e.g. seasonal or male status effects. In addition, by this paired design we were able to control for effects of different source bird song. The simulated territorial intrusion (STI) experiments were performed by placing a stuffed decoy (male in full adult plumage protected by an inconspicuous cage, three different decoys used) into the center of the respective territories. A remote-controlled loudspeaker (Foxpro Scorpion, digital game caller, FOXPRO Inc. Lewistown, USA) was put underneath the decoy to play back the territorial song of a potential rival at a sound pressure level of 65 dB SPL at 1 m (as measured with a CEL 573.B1 Sound Level Analyzer). We only started an experiment when a male was singing. The song was recorded 10 min prior to the start of the STI, during the 20 min STI and 10 min after the STI with a Sennheiser directional microphone (ME66/K6) connected to a Marantz solid state recorder PMD 660 (sampling frequency: 44.1 kHz; resolution: 16 bit).

## Data Analysis

The song was analyzed using Avisoft-SASLab Pro software, version 4.51. Recordings were visualized in spectrograms (settings: sample rate 22,050 Hz, FFT = 256 points, Hamming-window, overlap: 50%). We determined the number of songs by visual inspection and selected songs of sufficient quality (low background noise) for further sound analysis. Each song of black redstarts can be divided into three distinct parts (part A, B and C, see Fig. 4.1 and e.g. (Cucco and Malacarne 1999)) with a pause of varying duration between part A and B. We measured the duration of parts A, B and C, the total song and the duration of pauses between A and B (Fig. 4.1). We counted the number of elements of part A and C (mean of max. 20 song renditions). We also determined the frequency bandwidth and the maximum frequency of part A, B and C using the automatic parameter measurement function (threshold −20 dB) in Avisoft (mean of max. 10 renditions of high-quality songs).

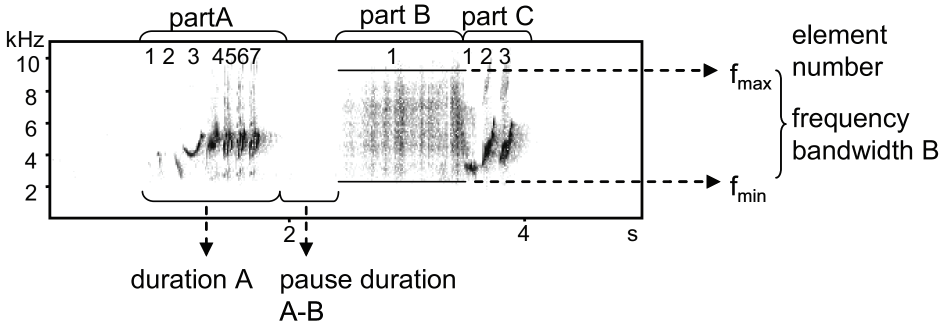


**Fig. 4.1 A song of a black redstart illustrating the acoustic measures analyzed** (Spectrogram: Avisoft-SASLab Pro, sample rate 22, 050 Hz, FFT=256 points, Hamming-Window, Overlap: 50%).

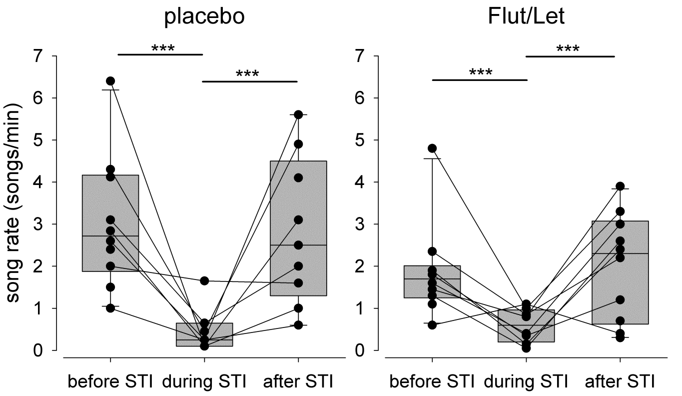
Data were analyzed with R version 2.9.1 (R Core Team 2014). Song before, during, and after the STI was analyzed using general linear mixed models with bird identity as a random effect to control for repeated measures. We analyzed whether the dependent variables (number of songs, song duration, duration of part A, B, C and the pause between part A and B, the number of elements in part A and C and maximum frequency and bandwidth of all parts) were influenced by the Flut/Let treatment, the context (testing phase of the STI) and their interaction. In all cases, dependent variables where transformed if assumptions of normality and/or equality of variances were not met. Significance was accepted at α ≤0.05 (two-tailed).

# Results

## Song in Spring

Males sang significantly fewer songs during the STI than when singing spontaneously before and after the STI (Fig. 4.2a, Table 4.1). Song duration significantly changed in placebo-implanted males, with songs during the STI being shorter than before or after the STI. In Flut/Let-males, song duration remained constant before, during and after the STI (Table 4.1). The shortening of the song in placebo-implanted males was mainly due to a significantly shorter pause between song part A and B (Table 4.1), because the durations of the three song parts (A, B and C) did not differ significantly before, during and after the STI (Table 4.1).

A) breeding season (spring)



B) nonbreeding season (fall)

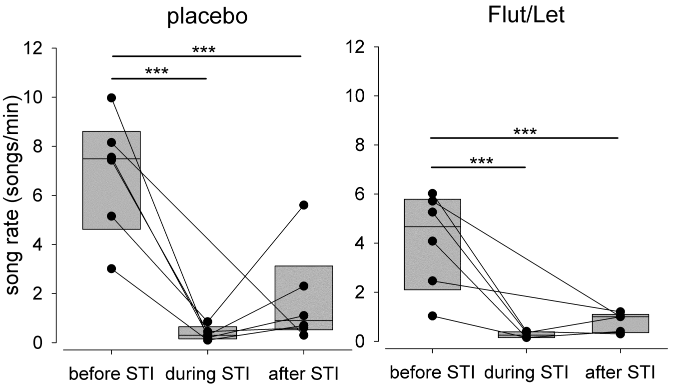


**Fig. 4.2 Song rate before, during and after the STI**. Depicted separately for males treated with flutamide and letrozole (‘Flut/Let’) and placebo treated males (‘placebo’) in A) spring (n=10 per group) and B) in fall (n=6 per group). Each circle represents one individual male and measurements of the same male are connected by a line. Asterisks indicate significance (*** p<0.001) and are according to a priori set contrasts (before vs. STI and before vs. after the STI). Mind the different scales in A and B.

**Table 4.1 Linear mixed model results for the effects of context and Flut/Let-treatment on song output and structure in spring.**

| **element** | **treatment** | **context** | **interaction** | **Coheńs d [95% CI]** | |
|---|---|---|---|---|---|
| | | | | **placebo** | **Flut/Let** |
| song rate | F1,18=2.3 | **F2,30=23.6** | F2,30=1.3 | **2.0** | **1.4** |
| | p=0.1 | **p<0.0001** | p=0.3 | **[0.8, 3.2]** | **[0.4, 2.5]** |
| song duration | F1,18=0.2 | **F2,30=6.7** | **F2,30=3.8** | **1.5** | 0.1 |
| | p=0.6 | **p=0.004** | **p=0.03** | **[0.3, 2.5]** | [−0.9, 1.0] |
| duration A | F1,18=0.7 | F2,30=2.3 | F2,30=1.4 | 0.2 | −1.0 |
| | p=0.4 | p=0.1 | p=0.3 | [−0.7, 1.2] | [−2.0, 0.01] |
| duration B | F1,18=0.2 | F2,30=2.0 | F2,30=0.4 | −0.3 | −0.4 |
| | p=0.6 | p=0.2 | p=0.6 | [−1.3, 0.7] | [−1.4, 0.5] |
| duration C | F1,18=0.4 | F2,30=0.9 | F2,30=0.2 | 0.7 | −0.1 |
| | p=0.6 | p=0.4 | p=0.8 | [−0.3, 1.7] | [−1.1, 0.8] |
| duration pause A-B | F1,18=1.2 | **F2,30=7.6** | F2,30=1.0 | **1.2** | 0.6 |
| | p=0.3 | **p=0.002** | p=0.4 | **[0.1, 2.2]** | [−0.4, 1.5] |
| no. of elements in A | F1,18=0.6 | **F2,30=23.1** | F2,30=1.1 | **−2.6** | **−1.7** |
| | p=0.6 | **p<0.0001** | p=0.4 | **[−3.9, −1.3]** | **[−2.8, −0.6]** |
| no. of elements in C | F1,18=0.04 | **F2,30=12.2** | F2,30=0.9 | **−1.9** | −0.8 |
| | p=0.8 | **p<0.0001** | p=0.4 | **[−3.0, −0.7]** | [−1.8, 0.2] |
| freq bandwidth A | F1,18=1.5 | F2,30=1.1 | F2,30=2.1 | 0.01 | 0.7 |
| | p=0.2 | p=0.4 | p=0.1 | [−1.0, 1.0] | [−0.3, 1.6] |
| max frequency A | F1,18=1.4 | **F2,30=3.9** | **F2,30=5.1** | −0.1 | **1.4** |
| | p=0.3 | **p=0.03** | **p=0.01** | [−1.0, 0.9] | **[0.3, 2.4]** |
| freq bandwidth B | F1,18=3.7 | **F2,30=4.4** | **F2,30=5.4** | **−1.4** | −0.1 |
| | p=0.07 | **p=0.02** | **p=0.009** | **[−2.4, −0.3]** | [−1.0, 0.9] |
| max frequency B | **F1,18=6.6** | **F2,30=3.6** | F2,30=2.0 | **−1.0** | −0.4 |
| | **p=0.02** | **p=0.04** | p=0.2 | **[−2.1, 0.0]** | [−1.3, 0.6] |
| freq bandwidth C | F1,18=0.1 | F2,30=0.9 | F2,30=0.09 | −0.2 | 0.02 |
| | p=0.7 | p=0.4 | p=0.9 | [−1.2, 0.8] | [−0.9, 1.0] |
| max frequency C | F1,18=0.2 | F2,30=2.1 | F2,30=0.2 | 0.1 | 0.2 |
| | p=0.7 | p=0.1 | p=0.8 | [−0.9, 1.0] | [−0.7, 1.2] |

Context is a within-subjects factor with three levels: before STI (spontaneously sung songs), during STI (playback and decoy present) and after STI (directly after removal of playback and decoy). Treatment is a between-subjects factor with two levels: placebo-implanted vs. blocker-implanted males. To control for repeated measures the ID of each territory owner was included as random intercept. Significant results are highlighted in bold.

Both placebo-implanted and Flut/Let-males sang significantly more elements in song parts A and C during and after the STI than before the STI (Table 4.1, Figs. 4.3c, d). This element increase resulted from an increase in the number of elements of the trilled phrases of part A or C, respectively (Fig. 4.1). By definition, part B did not change with respect to this measure because it consisted of one element only (Fig. 4.1).

Flut/Let-males sang part A with a significantly lower maximum frequency during and after the STI than before the STI. In contrast, the maximum frequency of part A did not change before, during and after the STI in placebo-implanted males (Table 4.1, Fig. 4.3a). Both treatment groups sang part B with a significantly higher maximum frequency during the STI than before the STI. Furthermore, the maximum frequency of this part tended to remain high after the STI in placebo-implanted males but not in Flut/Let-males (Table 4.1). Consequently, placebo-implanted males sang part B with a significantly larger frequency bandwidth during and after the STI than before the STI, while frequency bandwidth of part B did not change in Flut/Let-implanted males (Table 4.1, Fig. 4.3b).

Maximum frequency and the frequency bandwidth of part C did not change in response to the STI or Flut/Let-treatment (Table 4.1).

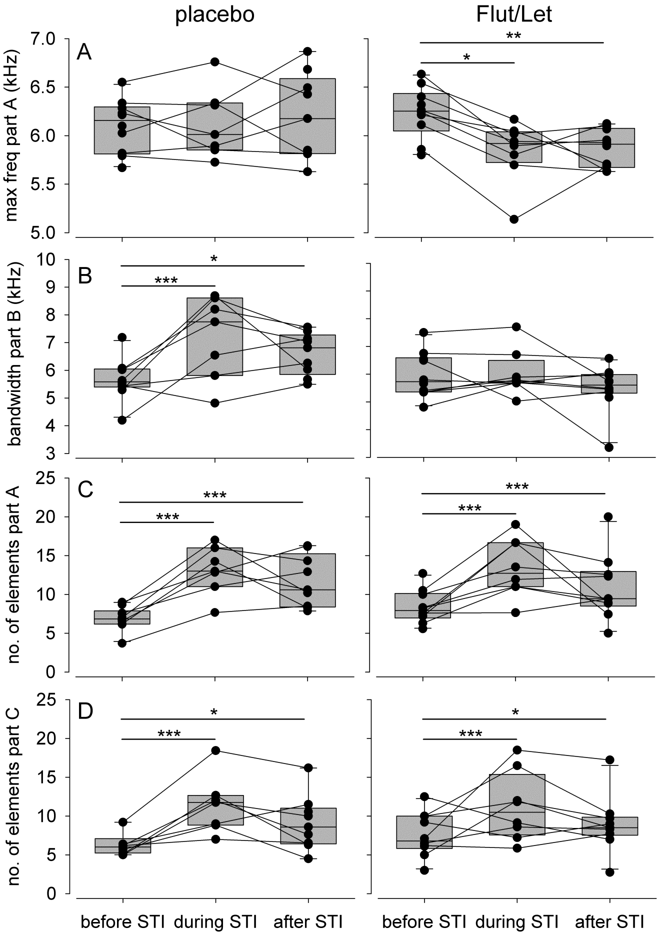


**Fig. 4.3 Structural song measures before, during and after the STI.** Depicted separately for males treated with flutamide and letrozole ('Flut/Let', n=10) and placebo treated males ('placebo', n=10) in spring. Each circle represents one individual male and measurements of the same male are connected by a line. Asterisks indicate significance (* $p<0.05$, ** $p<0.01$, *** $p<0.001$) and are according to a priori set contrasts (before vs. STI and before vs. after the STI).

## Song in Fall

In both treatment groups focal males sang fewer songs during and after the STI than before the experimental challenge (Fig. 4.2b, Table 4.2). Males of both treatment groups increased the number of elements in part A (Fig. 4.4c) and C (Fig. 4.4d) in response to the experimental challenge while decreasing the maximum frequency of part A (Fig. 4.4a) and decreasing the frequency bandwidth of part C (Table 4.2). Males sang part B with a significantly higher maximum frequency in response to the simulated territorial intrusion than during spontaneous song and this did again not significantly differ between placebo and Flut/Let-implanted males (Table 4.2). However, this effect is not reflected in a higher frequency bandwidth of part B in fall, in contrast to placebo-implanted males in spring (Fig. 4.4b, Table 4.2). Furthermore, changes in the frequency bandwidth of part B occur at a far narrower range in fall than in spring (Figs. 4.3b and 4.4b). With regard to the effect sizes (Table 4.2) we suggest to treat the results on frequency measures in fall with caution.

**Table 4.2 Linear mixed model results for the effects of context and Flut/Let treatment on song output and structure in fall.**

| element | treatment | context | interaction | Coheńs d [95%CI] placebo | Flut/Let |
|---|---|---|---|---|---|
| song rate | F1,10=3.0 | **F2,18=43.1** | | **2.3** | **2.4** |
| | p=0.1 | **p<0.0001** | | **[0.8, 3.8]** | **[0.8, 3.9]** |
| song duration | F1,10=0.02 | F2,18=2.1 | | 0.8 | 0.1 |
| | p=0.9 | p=0.1 | | [−0.4, 2.9] | [−1.0, 1.2] |
| duration A | F1,10=4.3 | F2,18=3.2 | | 0.3 | −1.3 |
| | p=0.06 | p=0.06 | | [−0.8, 1.5] | [−1.3, 1.0] |
| duration B | F1,10=0.01 | F2,18=0.7 | | 0.7 | 0.05 |
| | p=0.9 | p=0.5 | | [−0.5, 1.8] | [−1.1, 1.2] |
| duration C | F1,10=2.2 | F2,18=3.4 | | −0.1 | −0.7 |
| | p=0.2 | p=0.06 | | [−1.2, 1.0] | [−1.9, 0.5] |
| duration pause A-B | F1,10=0.4 | F2,18=0.1 | | −0.2 | 0.6 |
| | p=0.5 | p=0.9 | | [−1.3, 1.0] | [−0.6, 1.7] |
| no. of elements in A | F1,10=2.4 | **F2,14=11.9** | F2,14=3.8 | **−1.6** | **−2.6** |
| | p=0.2 | **p<0.001** | p=0.05 | **[−3.0, −0.1]** | **[−4.1, −0.9]** |
| no. of elements in C | F1,10=0.2 | **F2,16=26.1** | | **−4.2** | **−2.3** |
| | p=0.2 | **p<0.0001** | | **[−6.4, −1.9]** | **[−3.7, −0.7]** |
| freq bandwidth A | F1,10=0.2 | F2,16=2.0 | | 0.6 | 2.1 |
| | p=0.7 | p=0.2 | | [−0.6, 1.8] | [0.5, 3.6] |
| max frequency A | F1,10=0.08 | **F2,16=3.9** | | 0.7 | 0.9 |
| | p=0.8 | **p=0.04** | | [−0.6, 1.9] | [−0.3, 2.1] |
| | | | | **combined: 1.0 [0.1,0.9]** | |
| freq bandwidth B | F1,10=0.7 | F2,13=3.2 | | −1.6 | −0.6 |
| | p=0.4 | p=0.08 | | [−3.0, −0.2] | [−1.8, 0.6] |
| max frequency B | F1,10=1.5 | **F2,13=5.0** | | **−1.9** | −0.8 |
| | p=0.2 | **p=0.02** | | **[−3.3, −0.4]** | [−2.0, 0.5] |
| freq bandwidth C | F1,10=0.03 | **F2,16=3.6** | | 1.0 | 0.5 |
| | p=0.9 | **p=0.05** | | [−0.3, 2.3] | [−0.7, 1.6] |
| max frequency C | F1,10=0.1 | F2,16=2.2 | | 0.8 | 0.3 |
| | p=0.7 | p=0.1 | | [−0.5, 2.9] | [−0.9, 1.4] |

Context is a within-subjects factor with three levels: before STI (spontaneously produced songs), during STI (playback and decoy present) and after STI (directly after removal of playback and decoy). Treatment is a between-subjects factor with two levels: placebo-implanted versus blocker-implanted males. Significant results are highlighted in bold.

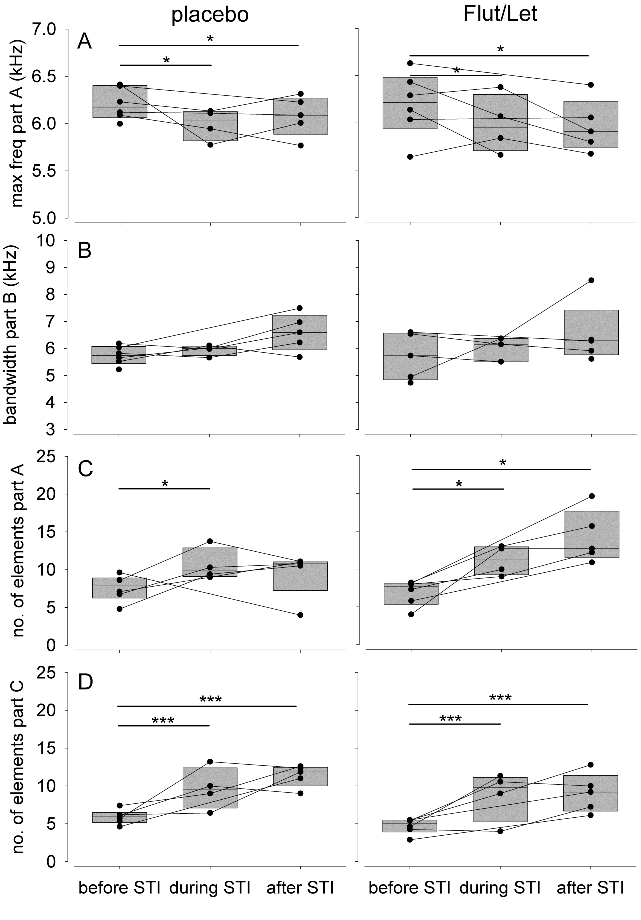


**Fig. 4.4 Structural song measures before, during and after the STI.** Depicted separately for males treated with flutamide and letrozole ('Flut/Let', n=6) and placebo treated males ('placebo', n=6) in fall. Each circle represents one individual male and measurements of the same male are connected by a line. Asterisks indicate significance (* p<0.05, *** p<0.001) and are according to a priori set contrasts (before vs. STI and before vs. after the STI).

# Discussion

In this study, we explored the role of testosterone (and its estrogenic metabolites) in modulating song characteristics of black redstarts in a spontaneous and a reactive context both during breeding and non-breeding. Territorial males of both treatment groups and in both seasons did change structural song parameters in an aggressive context. In spring, both treatment groups increased the number of elements sung in parts of their song in response to the STI. However, Flut/Let males decreased the maximum acoustic frequency of one song part in response to the STI, while placebo-implanted males kept this acoustic measure constant throughout the challenge. Furthermore, placebo-implanted males sang the atonal part of their song with a broader frequency range. In contrast to Flut/Let males, placebo-implanted males increased signal density by singing shorter songs with shorter pauses between song parts in the STI. In summary, these results provide a good example of the activational role of testosterone not only on song activity in general, but also on the specific singing style depending on the context.

The results of this study indicate that song sung during a territorial encounter is of higher competitive value than song sung in an undisturbed situation and may, therefore, convey information about the motivation or quality of the territory holder. During simulated intrusions in fall, when testosterone levels are naturally low in this species, males of both treatment groups sang similar to Flut/Let-implanted males during breeding. We conclude that these changes in song in response to a simulated territorial intruder were influenced by the Flut/Let treatment and by season: structural changes in song were less pronounced in Flut/Let males and in all males during non-breeding in fall compared to placebo-implanted males in spring.

## Song Modulation during Territorial Challenges

Black redstarts of both treatment groups in spring sang more elements in parts A and C and placebo-implanted birds increased the frequency bandwidth of part B when a simulated rival intruded the territory. Additionally, Flut/Let males decreased the maximum frequency of part A. These structural song parameters have been suggested to be physically challenging in other species (reviewed in (Podos et al. 2009)). Also, with regard to trilled parts, it has been suggested previously that the production of repeated (trilled) syllables with a high frequency bandwidth is challenging (reviewed in (Podos et al. 2009)). For example, in swamp sparrows, male age, size, and early developmental conditions correlated with these song characteristics, and can therefore serve as honest signals of male quality (Searcy et al. 1997; Illes et al. 2006; Ballentine 2009). Females of some species prefer songs sung with a high trill rate and broad frequency bandwidth (Drăgănoiu et al. 2002; Ballentine et al. 2004).

Furthermore, swamp sparrows increase both trill rate and frequency bandwidth in response to simulated territorial intruders (DuBois et al. 2009).

Even though songs of control males were shorter during the STI than before (which might occur counter-intuitive at first, since usually birds increase song output when challenged), this resulted in a higher signal density. Increasing the signal density by changing the song output in an aggressive context seems to be a common strategy among bird species (e.g. (Galeotti et al. 1997; Beckett and Ritchison 2010). In our study on black redstarts, this increase was realized by a shortening of pauses between song parts.

## Treatment and Season Affect Song Modulation during Territorial Challenges

Although all males (regardless of treatment and season) changed their song in the aggressive context, Flut/Let males in spring and all males challenged during non-breeding in fall did so to a lesser extent than placebo males during breeding in spring. The changes that we find to be inhibited by the Flut/Let-treatment in spring (i.e. maximum frequency of part A and frequency bandwidth of part B) are similar to the parameters Cucco and Malacarne (Cucco and Malacarne 1999) found to be characteristic for adult males song as opposed to yearling males' song. These parallels in acoustic features that differ between age-groups (Cucco and Malacarne 1999) and males of different hormonal status (our study) deserve further consideration. Yearlings as well as males with low testosterone levels might fail to produce challenging acoustic features due to lack of experience. Considering that adult male black redstarts (singing 'mature song') in general have a higher reproductive success than yearlings (Landmann and Kollinsky 1995b; Landmann 1996), we assume that this mature song is indicating a better quality and our Flut/Let-implanted males failed to produce this 'mature song'. Thus, context-dependent changes in song structure may indeed reveal information about the quality of the producer.

In Flut/Let-implanted males during spring and all males during fall the increase in the number of elements in part A was associated with a decrease in its maximum frequency. Therefore, Flut/Let-males in spring and all males in fall tended to sing this song part with a lower frequency bandwidth during a challenge than during spontaneous song. This might be interpreted as a failure to increase the number of elements and maintain the frequency at the same time in terms of a performance constraint, or alternatively, that Flut/Let-implanted males invested less into the production of these signals than did placebo-implanted birds in spring. Considering that territorial behaviors other than song were not affected by a Flut/Let-treatment in spring (Apfelbeck et al. 2013a) it is likely that motivational differences can not exclusively account for our results.

In addition, in contrast to placebo-implanted males Flut/Let males did not increase the frequency bandwidth of song part B, which consists only of a single noisy song element. Noisy elements are characterized as atonal, non-harmonic sounds occupying a range of frequencies (Fig. 4.1). There are good reasons to assume that such atonal song elements are not produced by the syrinx but by modulating the airflow in the vocal tract (reviewed in (Hoese et al. 2000)). Accordingly, placebo-implanted birds may sing with a higher air pressure and thus louder than Flut/Let-males. As a consequence, a broader range of frequencies is "broadcasted" in placebo-implanted males than in Flut/Let-males. Alternatively, a broader frequency range might be achieved by an increase in beak opening (e.g. (Hoese et al. 2000)). In barn swallows, the song characteristics of a similar harsh or noisy element, the rattle, were correlated with testosterone concentration (Galeotti et al. 1997). Changing the acoustic properties of such elements within limits may be interpreted as a way to increase their signal value as described in the framework of index signals.

Our results obtained in breeding and non-breeding males suggest that some, though not all song response measures in an aggressive context are mediated by testosterone or estradiol. Blocking these hormones particularly affected structural song measures. This may indicate that testosterone represents an underlying mechanism allowing the modification of 'index signals' such as trill rate or frequency measures. Similar results were recently reported for singing mice (Pasch et al. 2011) and may thus reflect a general mechanism in vertebrates. In birds, such a modification within limits may be achieved for example by modifying properties of the syrinx, an organ that is sensitive to testosterone and estradiol (Wade and Buhlman 2000; Veney and Wade 2004, 2005), or the beak muscles (Hoese et al. 2000). In addition, the neuronal coordination of singing might be affected by changes in testosterone levels, too (reviewed in (Balthazart et al. 2010)). Studies on male Gambel's white-crowned sparrows (*Zonotrichia leucophrys Gambelii*) did provide evidence that decreasing or increasing testosterone levels in the brain can affect the nuclei of the song control system within very few days or even hours (Thompson et al. 2007; Thompson and Brenowitz 2010). Furthermore, in some species testosterone levels rise during territorial disputes (reviewed in (Goymann et al. 2007a; Goymann 2009) and this increase in testosterone may affect the behavior and persistence of the male during or after the challenge (Wingfield 1994b; Oyegbile and Marler 2005). However, black redstarts do not show such a short-term increase in testosterone levels during territorial intrusions (Apfelbeck and Goymann 2011; Apfelbeck et al. 2011). Thus, any effects of testosterone on song and territorial behavior probably take place as a consequence of the increase in testosterone at the beginning of the breeding season (i.e. an activational effect).

Furthermore, these context-dependent changes in song may be regulated by aromatase activity in the pre-optic area (Riters and Ball 1999; Riters et al. 2000) or changes in androgen and estrogen sensitivity in the song control nucleus HVC (Gahr and Metzdorf 1997) because redstarts show a higher expression of aromatase mRNA in the pre-optic area in spring than in fall, but no seasonal change in HVC size (Apfelbeck et al. 2013b).

### Conclusions

Our study demonstrates that blocking the actions of testosterone affected both song output and structural song measures of black redstarts during competitive situations during the breeding season, but not outside the breeding season. We conclude that testosterone may affect both the signal parameters indicating the motivation and/or the ability to engage in competitive interactions such as territorial disputes. This might be achieved by effects of testosterone on the neuronal and physiological capabilities to produce certain song elements depending on the behavioral context. This very nicely illustrates that hormones can change the likelihood of a behavior, often in a context-dependent manner.

## Acknowledgements

We thank Erwin Nemeth, Henrik Brumm, Gabriel Beckers, Sue-Ann Zollinger and Manfred Gahr for valuable discussions about the study, Alexander Weber for assisting in the field, and two anonymous referees for providing constructive criticisms on earlier versions of the manuscript. The study was kindly funded by the Max-Planck Gesellschaft and the ‘Berliner Programm’ of the Freie Universität Berlin (research travel grants to S. Kiefer and KGM). Novartis kindly provided the authors with letrozole. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

# CHAPTER 5 Different pace of life, but similar testosterone levels and breeding behavior in temperate and afrotropical Stonechats

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# Abstract

Avian life histories vary with latitude in a predictable way: tropical birds live a slower pace of life than temperate ones, i.e. they usually get older but produce fewer young during a single breeding season. The physiological mechanisms of a slow pace of life in the tropics are not very well understood, but it is thought that the sex steroid testosterone may play a central role. In the Northern hemisphere, birds need to adjust their life history to environmental seasonality. In the absence of strong seasonality, such as the nearly constant environments of tropical rain forests, many birds can potentially breed year-round, thereby typically expressing low levels of territorial behaviour and low levels of testosterone. We investigated differences in testosterone levels and territorial behaviour in stonechats *(Saxicola torquata spp.)*. Remarkably, they breed seasonally in temperate and in afrotropical environments. Tropical stonechats followed a slower pace of life than temperate stonechats. However, this was not reflected in their reproductive physiology: in both tropical and temperate stonechats testosterone was elevated during breeding. Furthermore, tropical stonechats responded as vigorously to a simulated territorial intruder as temperate stonechats with the strongest response during nestbuilding when testosterone levels were highest and females fertile. In addition, tropical males increased their peak song frequency during nestbuilding. Although migratory temperate stonechats sang with a higher song rate than resident tropical stonechats during mating, they did not have higher testosterone levels during that period.

Thus, in stonechats, the seasonal profile of testosterone does not seem to differ between tropical and temperate populations. We suggest that in seasonally breeding tropical species, testosterone activates territorial behaviour and facilitates mate guarding during the fertile period of females similar to temperate species. Therefore, testosterone does not hold as a general mediator of differences in pace of life between tropical and temperate species. In light of the seasonal diversity of tropical environments, the pace of life theory and its underlying endocrine control mechanisms should be reconsidered in future studies.

# Introduction

In contrast to environments of the northern hemisphere where temperature and daylength change drastically over the year, environmental conditions in tropical biomes are relatively stable year-round (Olson et al. 2001; Goymann and Helm 2014). These stable environments seem to have allowed species to live a slower "pace of life" than species living at higher latitudes (Promislow and Harvey 1990; Ricklefs 2000). This is especially well studied in birds and it has been shown that avian life histories vary with latitude in a predictable way: tropical birds produce smaller clutches of offspring (Jetz et al. 2008), have a lower basal metabolic rate (Wiersma et al. 2007), but live longer (Peach et al. 2001; Møller 2007) than temperate birds. As time, nutrients, and energy are limited resources reproduction and longevity cannot be maximized simultaneously and a trade-off arises (Stearns and Roff 1992). While the difference in clutch size at different latitudes is well established and holds on a global scale (Jetz et al. 2008), we know little about the physiological mechanisms that mediate this variation. In males, one potential key candidate is testosterone (Hau 2007). This sex steroid is the major hormone that regulates reproduction in male vertebrates (Nelson 2005). Testosterone has pleiotropic effects and boosts male fecundity by activating spermatogenesis, secondary sex characteristics and sexual and aggressive behaviours associated with breeding (Lincoln et al. 1972; Ball and Balthazart 2004; Nelson 2005). On the other hand, prolonged high levels of testosterone come at a cost as males with high testosterone levels tend to invest less into self-maintenance and their immune system (Malo et al. 2009), pay less attention to predators, are prone to get injured during fights, and reduce parental behaviours (Schwagmeyer et al. 2005); all of which can reduce survival probability and longevity (Marler and Moore 1989). Experimental studies within populations show that testosterone can shift the balance between reproduction and self-maintenance and survival towards mating effort (Mills et al. 2009). Furthermore, testosterone levels do not only vary substantially between individuals within a population, but also between species. (Wingfield et al. 1990; Hirschenhauser et al. 2003; Garamszegi et al. 2008; Goymann 2009). Thus, quantitative differences in testosterone levels (both peak and duration of the peak) may be plausible mediators of differential reproductive investment of tropical and temperate species. In agreement with this hypothesis, interspecific variation in testosterone levels seems to follow a latitudinal trend along the pace of life axis: tropical species tend to have lower testosterone levels than species breeding at higher latitudes (Garamszegi et al. 2008). In a comparative study between neotropical rainforest and North American temperate songbirds, the tropical species indeed had the lower breeding season testosterone levels (Hau et al. 2010). Furthermore, many tropical species seem to lack a peak in testosterone at the beginning of the breeding season (Levin and Wingfield 1992; Wikelski et al. 2003a; Fedy and Stutchbury 2006) that is characteristically found in temperate socially monogamous birds and associated with territory establishment and mate acquisition. Surprisingly, compared to temperate species hormonal studies in tropical birds are scarce and most of them have been conducted in lowland rainforest species. Many of these species have extended breeding seasons and low breeding synchrony between pairs (Stouffer et al. 2013). Under these circumstances, high testosterone levels may not be necessary as competition between males and mate guarding is low (Stutchbury and Morton 2008). However, many tropical biomes are characterized by distinct dry and rainy seasons (Olson et al. 2001; Goymann and Helm 2014) and conditions for breeding are only suitable during a certain time of the year. In addition, although many tropical birds are resident, short-distance and altitudinal migration is common (Alerstam 1993). Few studies indicate that tropical songbirds breeding in seasonal habitats have similar annual patterns of testosterone as temperate species ((Moore et al. 2004; Goymann et al. 2006; Addis et al. 2010); reviewed in (Goymann et al. 2004; Goymann and Landys 2011)). Males breeding in seasonal tropical environments, may face the same reproductive challenges as males breeding at temperate latitudes (Fedy and Stutchbury 2006)with high competition between males especially at the beginning of the breeding season making elevated testosterone levels advantageous.

We are not aware of studies that have compared territorial behaviour between tropical and temperate species. In seasonal temperate breeders territorial behaviour has three major non-exclusive functions: resource defence, mate attraction (own and extrapair), and protection of paternity (Class and Moore 2011). Song, especially during dawn chorus, plays a key role in the communication of all these activities (e.g. (Kunc et al. 2005)). In species, in which the main function of territorial behaviour is to attract a mate and to protect paternity, song activity peaks during mating, the period of maximum fecundity in females (Bukacinska and Bukacinski 1994; Osiejuk and Kuczynski 2000; McDonald 2001; Bruni and Foote 2014). In species in which the intensity of territorial behaviour is relatively constant throughout the breeding season, it is probably more important for establishing and maintaining stable relationships with neighbouring males (Kunc et al. 2005). Territorial behaviour in tropical species is much less well studied. Although many tropical species are territorial year-round, the rate of dawn song and the intensity of territorial aggression towards intruders differ between breeding and nonbreeding seasons and breeding stages in some species (Dittami and Gwinner 1985; Hau et al. 2000; Addis et al. 2010; Class and Moore 2011; Chiver et al. 2015). However, as they are less time-constrained than migratory temperate birds (Velmala et al. 2015), occupy the same territories year-round and often have more stable relationships with their partners (Gill and Stutchbury 2006), competition for territories and mates may be less intense during breeding in socially monogamous tropical compared to temperate seasonal breeders. To clarify the role of variation in testosterone and territorial behaviour in life history variation between species clearly more studies on tropical species from various tropical biomes and comparative studies of temperate and tropical species are needed.

Our study system, the stonechat species complex Saxicola torquata spp. has played a major role in the generation of the pace of life theory (Ricklefs and Wikelski 2002). For example, common garden experiments have shown that tropical stonechats have a genetically fixed smaller clutch size (Gwinner et al. 1995a; Wikelski et al. 2003b) and a lower metabolic rate than their higher latitude relatives. East African stonechats generally lay a single clutch (but replacement clutches are common (Scheuerlein 2000; Urquhart 2010)), while European stonechats produce up to three consecutive clutches (Flinks and Pfeifer 1987; Urquhart 2010). East African stonechats are residents or occasional altitudinal migrants and stay with the same partner year round, while European stonechats are migratory (Dittami and Gwinner 1985; Urquhart 2010). Still, stonechats are socially monogamous seasonal breeders and defend a breeding territory regardless of latitude. Stonechats are thus an ideal model system to study the physiological mechanisms underlying differences in pace of life in a phylogenetically controlled setting.

Various previous studies have investigated testosterone levels in stonechats, but results were inconsistent and restricted to few populations. Stonechats in a temperate German population showed a similar profile of breeding season testosterone levels as other temperate breeders (Schwabl et al. 2005). Stonechats from Kenya had low year-round testosterone levels in one study in the field (Dittami and Gwinner 1985) and in one study in captivity (Rödl et al. 2004), but a peak in testosterone levels during nestbuilding in a different study (Goymann et al. 2006). The latter study suggests that despite a slow pace of life, tropical stonechats may be capable of high testosterone levels.

To examine these earlier results and specifically to advance our understanding of potential proximate causes of high testosterone levels in tropical birds, we studied several stonechat populations in East Africa and Europe and collected information about clutch size, age structure, male territorial behaviour (determined as territorial response to an intruder and song activity) and male testosterone levels during different breeding stages. As tropical stonechats invest less into reproduction than their temperate relatives we predicted, that stonechats from East African populations show less intense territorial behaviour and lower testosterone levels, but with a potential peak during nestbuilding, than stonechats from European populations.

# Material and Methods

## Study populations

Samples were collected from several closely related stonechat species in tropical East Africa (9 populations; 173 individuals; *Saxicola torquata axillaris*; altitudinal range: 1376 – 2500 m asl; in the years 2012 – 2013, fig. 5.1) and in Europe (6 populations; 201 individuals; *Saxicola torquata rubicola*, in the years 2009 – 2013, fig. 5.1). In East Africa, samples were obtained during breeding (n = 7 populations) and nonbreeding (n = 1 population), while in Europe samples were only obtained during the breeding season (n = 6 populations). East African resident populations of *S. t. axillaris* breed at different times of the year correspondent to the two major rainy seasons. Southern populations breed from October to December, while Northern populations breed from March to July. European stonechats breed from March to July. Breeding stage was determined through careful observation of pairs before capture (e.g. singing activity, nestbuilding activity by female, feeding activity of nestlings, presence of fledglings). In some cases, we were able to catch the female and breeding stage could be ascertained by the presence or absence of a brood patch. For located nests, we recorded the number of eggs or nestlings and checked the nest again after a few days. We recorded geographical location and altitude using a GPS device. We determined the age of individuals (yearling or adult (≥ 2years) by the moult pattern of the wings (Flinks 1994) and a picture of the wing was taken for later reference. All birds were measured (body mass, tarsus length and wing length), checked for moult, ringed with a numbered aluminium ring and a combination of three colour rings and released back into their territories. As expected from previous studies (Klaassen 1995), stonechats from East Africa were larger (tarsus: LRT: $\chi 2 = 5.9$, df = 1, p = 0.01, Bayesian estimates and credible intervals: intercept (East Africa): 22.9 [22.8, 23.1], difference from intercept: Europe -0.4 [-0.7, -0.1]; wing: LRT: $\chi 2 = 21.4$, df = 1, $p < 0.001$, intercept (East Africa): 71.0 [70.3, 71.8], Europe: -3.7 [-5.0, -2.4]) and heavier (LRT: $\chi 2 = 23.5$, df = 1, $p < 0.001$, intercept (East Africa): 16.3 [15.9, 16.6]; Europe: -1.72 [-2.3, -1.2]) than stonechats from Europe. All experimental procedures were approved by the governmental authorities of the respective countries.

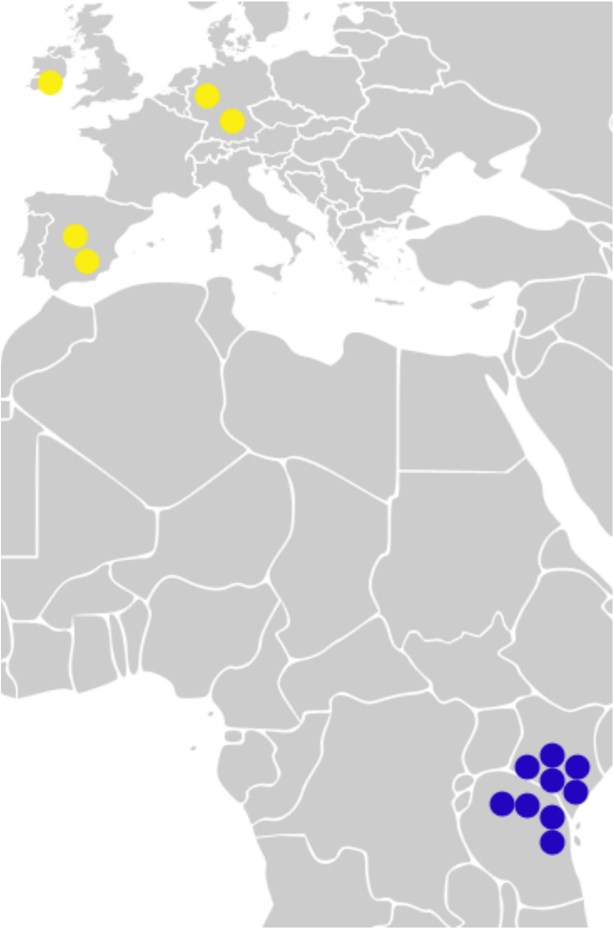

**Fig. 5.1 Approximate location of study sites in Europe (Ireland, Germany, Spain) and East Africa (Kenya, Tanzania).** Two populations in Germany are very close together and only shown as one dot. Colour of symbols codes for average clutch size (blue: 3 eggs, yellow: 5 eggs).

## Capture methods

Males were caught with baited spring traps and / or spring traps and a mounted decoy and playback (simulated territorial intrusion, STI) between 7:00 hrs and 18:00 hrs. In Europe we used playbacks downloaded from the British library or from www.xeno-canto.org. To assess if male stonechats modulate testosterone levels in response to simulated territorial intrusions we caught male stonechats passively or with playback and decoy in the German population throughout the breeding season. In East Africa, we usually used playback songs, which were recorded from the previously sampled population (see below). However, in some cases the stonechats did not respond to the playbacks from other populations and we continued to catch with baited spring traps only. Because of this discrepancy in trapping methods, we were not able to assess the effect of simulated territorial intrusions on testosterone levels in the East African populations.

## Plasma separation and hormone analysis

Immediately upon capture (228 ± 191 sec), a blood sample (~ 120 µl) was taken after venipuncture from the wing vein and collected into heparinized capillaries. Plasma was immediately separated by centrifugation with a Compur Minicentrifuge (Bayer Diagnostics) or a Spectrafuge Mini Laboratory Centrifuge (Labnet International, Inc.). The amount of plasma was measured with a Hamilton syringe and stored in 500 µl pure ethanol (Goymann et al. 2007b). After the end of each field season (≤ 4 months) samples were stored at -80°C. Testosterone concentration was determined by direct radioimmunoassay (RIA, following (Goymann et al. 2006)). Mean efficiency of the extraction with dichloromethane varied between 81 and 89 %. The lower limit of detection of the assay was determined as the first value outside the 95 % confidence intervals for the zero standard (Bmax) and was on average 0.35 ± 0.05 pg/tube. Samples were measured in duplicates in five assays, each containing samples from East African and European populations. The intra-assay coefficients of variation varied between 4.2 % and 15.1 %. The inter-assay variation was 11.3 %. As the testosterone antibody shows significant cross-reactions with 5a-dihydrotestosterone (44 %) our measurements may include a minor fraction of this additional androgen.

## Behavioural response to simulated territorial intrusions

For two populations in Europe (Germany) and four populations in East Africa we performed STI experiments to record the behaviour of male stonechats towards an intruder during breeding. To elicit a territorial response we placed a stuffed decoy into the centre of a territory (determined by singing posts of the male or nest location) and played back song. In Germany, we used 8 different playbacks in random order (wav.files, British library). In East Africa, we used four to six different playbacks recorded from the same population and took care to avoid songs from neighbouring males within hearing distance. As decoys we used stuffed males in full adult plumage that were protected by an inconspicuous cage. In Europe, we randomly assigned two decoys from Germany and in East Africa eight decoys from each of the two populations, Kinangop (Kenya) and Arusha (Tanzania), for each experiment. The behaviour of the male and its latency to attack were recorded for 10 min. The intensity of the behavioural response was determined as in (Schwabl et al. 2005). Males that did not attack the decoy were assigned an attack latency of 60 min.

## Song recording and analysis

We recorded 5 - 10 min of song from males of two European (time: 0800 – 1200, Germany, n = 30) and six East African populations (time: 0350 – 0600, Kenya and Tanzania, n = 32) using a Marantz PMD 661 solid state recorder (Osnabrück, Germany) and Sennheiser ME66/K6 directional microphones (Georgsmarienhütte, Germany). We analysed songs (sampling frequency: 44.1 kHz; resolution: 16 bit) using the software Avisoft Sound Analysis Pro, v5.1.09 (Raimund Specht, Berlin, Germany) to determine the song rate (average number of songs per minute over a five minute period), element rate (number of elements divided by song duration), as well as peak (frequency of the highest amplitude sound), minimum, and maximum frequencies. We used the automatic parameter measurements setup to obtain the minimum and maximum frequency values measured at a standard decibel threshold (here −20 dB, total option) below the peak in the power spectra (Zollinger et al. 2012). We compared song between East African and European stonechats during the mating period. Unfortunately, we had to combine the prenesting and nestbuilding stages due to missing detailed breeding information in European stonechats. However, we were able to record song during different breeding stages for African stonechats, i.e. during i) prenesting, ii) nestbuilding, iii) incubation (includes egglaying), and iv) parental stages.

## Statistical analysis

### *General Analysis*

Statistical analysis was performed with R 3.1.0 (R Core Team 2014) in R Studio. Data were analysed with linear mixed models with the R packages lme4 and arm. We graphically checked if model assumptions were met. In all models we initially included population location as random intercept. Models that contained breeding phase (see below) or breeding stage as an explanatory factor the early breeding phase or prenesting breeding stage were set as reference levels (intercept) of the models. The most parsimonious model was chosen by stepwise reduction of the full model and comparison of nested models with likelihood ratio tests (LRT). To calculate model predictions we chose a Bayesian approach and estimated the model parameters as the median of their posterior distributions, and the 2.5 % and 97.5 % credible intervals (CI).

### *Testosterone*

To ensure that residuals were normally distributed and to control for heteroscedasticity we log transformed testosterone levels prior to analysis. In a first step we analysed all testosterone samples (n = 360) including also individuals without exact breeding stage information to determine the effect of latitude on breeding season testosterone levels in stonechats. For this analysis, breeding stage was determined on the population level and categorized as early, medium or late depending on sampling date and exact breeding stage of most individuals. We will refer to this rough categorization as breeding phase from now on. As the relationship between breeding phase and testosterone may differ between tropical and temperate latitudes, we, included a latitude*breeding phase interaction in the model. Furthermore, we controlled for capture method, time of day at capture, body mass, and age. We then reduced the data set and included only individuals with identified exact breeding stage (n = 254). To match East African and European samples we restricted European samples to the first brood. As time of day at capture, capture method and body mass had no major influence on testosterone in the larger data set we did not include them in this model. Initially, we incorporated location as random intercept, but as the variance accounted for by location after including the exact breeding stage was zero, we calculated a simple linear model.

### *Dawn song*

To estimate differences in song rate and frequency parameters between breeding stages in East African stonechats we first included breeding stage as a factor with two levels (mating: prenesting and nestbuilding combined; parental: egglaying, incubation, nestling and fledgling stages combined). If the response variable differed significantly between these two levels, we ran another model with breeding stage as a factor with four levels (prenesting, nestbuilding, incubation (includes egglaying), parental).

### *Behaviour*

To analyse the behavioural response to STIs we categorized breeding stage as prenesting, nestbuilding, incubation (includes egglaying) and parental (nestlings and fledglings) because sample sizes were low for some stages (e.g. egglaying). The behavioural response score was poisson distributed with most birds reaching the highest score of five and much lower numbers of birds in all other scores. We therefore reversed the score (highest score now zero) and analysed the data with a quasipoisson linear model to account for overdispersion (dispersion parameter 1.9). When using quasipoisson as link function it is not possible to compare models with LRTs. We, therefore, fitted the models twice both with poisson and with quasipoisson as link functions and calculated LRTs for the poisson models (Bolker 2014).

# Results

## Breeding biology and age structure

We collected information on clutch size from three European populations and five East African populations (table 5.1). East African stonechats had significantly smaller clutches than European stonechats (table 5.1; latitude: $F_{1, 207} = 479.9$, $t = 21.91$, $p < 0.001$, $R^2 = 0.70$, slope 0.046 [0.038, 0.046]). In table 5.1, we have complemented our original data with data on clutch size from previous studies on stonechats. Similar to the global pattern recorded by Jetz and colleagues (Jetz et al. 2008), clutch size is largest in the long distance migratory populations of Siberia and decreases consistently in populations closer to the equator. Furthermore, of all breeding males caught in our study, 86 % (190 out of 222) were adults in East Africa, while only 31 % (62 out of 203; $\chi 2 = 133.2$, df = 1, $p < 0.001$) were adults in Europe.

## Testosterone

***Testosterone levels of East African and European stonechats with respect to breeding stage and season***

Breeding phase had a highly significant effect on testosterone levels (LRT: $\chi 2 = 31.9$, df = 2, $p < 0.001$). Testosterone levels were highest early in the breeding season and then declined gradually (table 5.2, see fig. 5.3 for a European population). This effect of breeding phase on testosterone levels was similar at different latitudes (LRT: $\chi 2 = 0.7$, df = 2, $p = 0.7$, table 5.2) and testosterone did not differ between latitudes (LRT: $\chi 2 = 2.7$, df = 1, $p = 0.1$). Time of day (LRT: $\chi 2 = 0.4$, df = 1, $p = 0.5$), capture method (LRT: $\chi 2 = 0.06$, df = 1, $p = 0.8$, table 5.2) and body weight (LRT: $\chi 2 = 0.1$, df = 1, $p = 0.7$, table 5.2) had no significant influence on testosterone levels. Age was marginally significant as yearling males had slightly lower testosterone levels than adult males (LRT: $\chi 2 = 4.7$, df = 1, $p = 0.03$, table 5.2).

Breeding stage still had a strong effect on testosterone levels when it was included as a factor on a finer scale (LRT: $\chi 2 = 41.7$, df = 5, $p < 0.001$, table 5.2, fig. 5.2) independent of latitude (interaction: LRT: $\chi 2 = 7.1$, df = 5, $p = 0.2$; latitude: LRT: $\chi 2 = 1.4$, df = 1, $p = 0.2$). Testosterone levels peaked during nestbuilding and were especially low during the nestling stage. In this reduced data set, age had no significant influence on testosterone levels (LRT: $\chi 2 = 2.06$, df = 1, $p = 0.1$).

In one East African population we also measured testosterone levels during the nonbreeding season (Arusha, April 2013). Testosterone levels of males caught outside the breeding season were much lower than during breeding and were close to the detection limit of the assay ($F_{1,26} = 22.8$, $p < 0.001$, $R^2 = 0.45$; means and CI: breeding: 0.6 ng/ml [0.4, 0.8], nonbreeding: 0.09 ng/ml [0.04, 0.18], table 5.2).

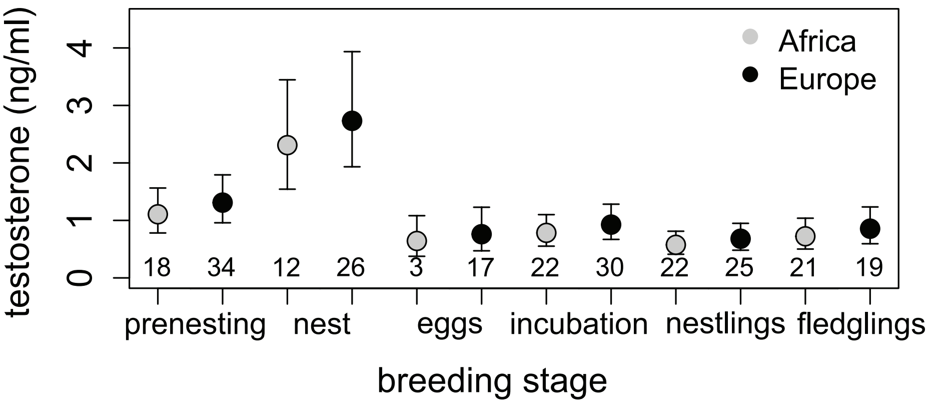

**Fig. 5.2 Testosterone levels of East African and European stonechats during different breeding stages of the first brood.** During all breeding stages East African and European stonechats had similar testosterone levels. Testosterone levels peaked during nestbuilding and found a trough when stonechats were feeding nestlings. Points represent posterior means and error bars 95 % credible intervals. Sample sizes are displayed below error bars.

**Table 5.1 Clutch size and number of broods of stonechats breeding at different latitudes.**

| **population** | **migration** | **latitude, longitude & altitude** | **no of nests** | **clutch size: mean ± se, (range)** | **broods** | **Ref.** |
|---|---|---|---|---|---|---|
| Central Siberia | long | **71°N** | 32 | **5.69**, (4 – 8) | 1 | Urquhart. 2002 |
| Kazakhstan | long | **52°N** | 522 | **5.90** ± 0.73 | 1 | Raess. 2006 |
| Ireland, Cork | partial | **51**°49`N, 8°00`W, 21 m asl | | **4.98** ± 0.1 (1.clutch)– **5.54** ± 0.1 (2.clutch) | 2 | Cummins et al. 2003 and this study |
| England | partial | **50° - 55°N** | | **5.06** – 5.3, (2 – 7) | 3 | Urquhart. 2002 |
| Germany, North Rhine Westphalia | short | **51**°47`N, 6°01`E, 15 m asl | 237; 112 | **5.24** ± 0.07, (3-7);<br>5.14 (4-6) (1.broods only) | 2-3 | Flinks et al. 1997 and this study |
| Slovakia | short | **49°N** | 258 | **5.40** ± 0.77 | 2-3 | Cummins. 2003 |
| Central Spain, Avila | short | **40**°32`N, 4°49`W, 1350 m asl | | 5-6 | 2 | pers comm JCI |
| Southern Spain, Seville | resident | **37**°39`N, 5°34`W, 57 m asl | | **4.19** ± 0.08, (3-6) | 2 | this study |
| Fuerteventura | resident | **28**°N | 128 | **2.7** ± 0.15 – **3.29** ± 0.1, (2-5) | 1-2 | Illera et al. 2006 |
| Kenya, Nakuru | resident | **0°S** | 39 | **3.1** ± 0.2, (2-5) | 1 | Dittami et al. 1985 |
| Kenya, Kinangop | resident | **0**°37' N, 36°29' E; 2470 asl | 21 | **3.2** ± 0.09, (3-4) | | this study |
| Tanzania, Arusha | resident | **3**°16`S, 36°51`E; 1573 m asl | 8 | **2.8** ± 0.18, (2-3) | 1-2 | this study |
| Tanzania, Monduli, | resident | **3**°14`S, 36°25`E; 1923 m asl | 30 | **2.8** ± 0.15, (2-4) | | this study |
| Tanzania, Usambara & Pare Mountains (combined due to small samples size) | resident | **4**°30`S, 38°13`E; 1533 m asl;<br>4°18`S, 37°57`E; 1563 m asl | 5 | **2.8** ± 0.18, (2-3) | | this study |

For study populations of the present study exact geographic locations are given, for all others studies only latitude is reported (bold numbers).

### *Testosterone levels in response to simulated territorial intrusions in European stonechats*

Male stonechats did not increase testosterone levels in response to STIs in any breeding phase (STI: LRT: $\chi2 = 0.6$, df = 1, p = 0.4; interaction: LRT: $\chi2 = 2.9$, df = 2, p = 0.24, table 5.2, fig. 5.3).

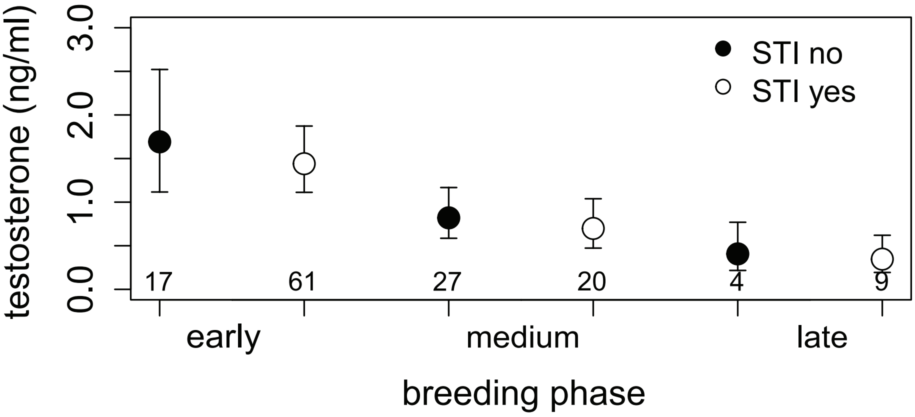

**Fig. 5.3 Testosterone levels of European stonechats in response to simulated territorial intrusions during different phases of the breeding season.** Male stonechats did not increase testosterone levels in response to STIs. Points represent posterior means and error bars 95 % credible intervals. Sample sizes are displayed below error bars.

**Table 5.2 Testosterone in relation to breeding stage, season and STI challenge.**

| **%** | **intercept (East Africa, prenesting, adults)** | **Europe** | **medium** | **late** | **yearling** | **weight** | **time of day** | **capture method** |
|---|---|---|---|---|---|---|---|---|
| 2.5 | 4.45 | - 0.12 | **- 0.78** | **- 1.62** | **- 0.59** | - 0.12 | - 0.0006 | - 0.3 |
| 50 | 6.92 | 0.39 | **- 0.46** | **- 1.19** | **- 0.31** | 0.02 | - 0.0001 | - 0.05 |
| 97.5 | 9.37 | 0.91 | **- 0.15** | **- 0.75** | **- 0.03** | 0.17 | 0.0003 | 0.21 |
|  | **intercept (East Africa, prenesting)** | **Europe** | **nest-building** | **egg-laying** | **incubation** | **nestlings** | **fledglings** |  |
| 2.5 | 6.65 | - 0.09 | **0.26** | - 1.13 | - 0.77 | **- 1.09** | - 0.85 |  |
| 50 | 7.0 | 0.18 | **0.7** | - 0.55 | - 0.35 | **- 0.65** | - 0.43 |  |
| 97.5 | 7.36 | 0.44 | **1.15** | 0.02 | 0.04 | **- 0.23** | 0.005 |  |
|  | **intercept (Arusha, breeding)** | **Arusha, nonbreeding** |  |  |  |  |  |  |
| 2.5 | 6.0 | **- 2.66** |  |  |  |  |  |  |
| 50 | 6.36 | **- 1.88** |  |  |  |  |  |  |
| 97.5 | 6.73 | **- 1.09** |  |  |  |  |  |  |
|  | **intercept (Europe, control, early)** | **Europe, STI** | **medium** | **late** |  |  |  |  |
| 2.5 | 7.05 | - 0.55 | **- 1.15** | **- 2.05** |  |  |  |  |
| 50 | 7.43 | - 0.16 | **- 0.73** | **- 1.42** |  |  |  |  |
| 97.5 | 7.81 | - 0.23 | **- 0.29** | **- 0.77** |  |  |  |  |

Rows show Bayesian estimates and credible intervals. When zero is not included within the credible intervals there is a ‘significant’ effect of this parameter on the dependent variable. Estimates of cofactors refer to differences from the intercept estimate. ‘Significant’ differences are shown in bold.

## Song

### *Song during the mating period of East African compared to European stonechats*

Several song traits differed between East African and European stonechats during the mating period (prenesting and nestbuilding). European stonechats sang with higher song rate (LRT: $\chi 2 = 8.9$, df = 1, $p < 0.001$), element rate (LRT: $\chi 2 = 14.8$, df = 1, $p < 0.001$), and peak frequency (LRT: $\chi 2 = 12.5$, df = 1, $p < 0.001$) than East African stonechats (table 5.3, fig. 5.4). The minimum (LRT: $\chi 2 = 1.4$, df = 1, p = 0.2), and maximum frequency (LRT: $\chi 2 = 3.4$, df = 1, p = 0.07), did not differ between East African and European stonechats (table 5.3, fig. 5.4).

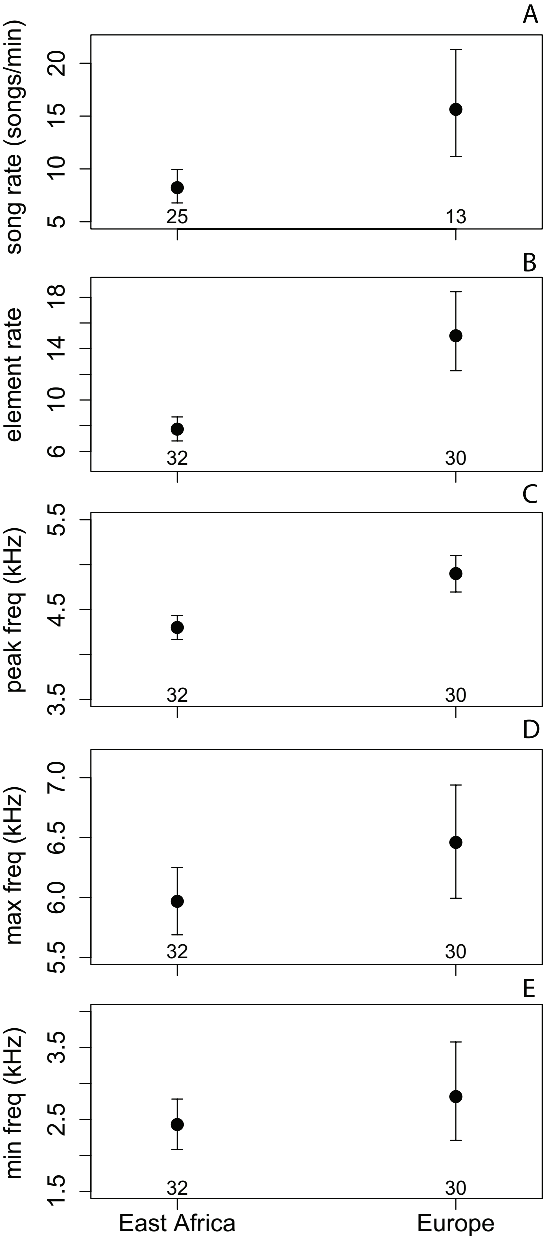

**Fig. 5.4 Song traits of East African and European stonechats with a) song rate, b) element rate per song, c) peak frequency, d) maximum frequency, and e) minimum frequency.** European stonechats sang with higher song rate, element rate, and peak frequency during the mating period than East African stonechats. Maximum frequency and minimum frequency were not significantly different. Points represent posterior means and error bars 95 % credible intervals. Sample sizes are displayed below error bars.

### *Changes in dawn song of tropical stonechats with breeding stage*

During dawn chorus, males sang significantly more and with a higher peak and minimum frequency during mating than during the parental stages (table 5.3, fig. 5.5, supplements). At a finer breeding stage resolution, males sang least, but with a higher peak frequency, during nestbuilding than during all other stages (although the credible interval for the difference between prenesting and nestbuilding for song rate crosses zero, table 5.3, fig. 5.5). The element rate and the maximum frequency did not differ between breeding stages (table 5.3, fig 5.5).

**Table 5.3 Song traits differed between East African and European stonechats during mating and in relation to breeding stage in East African stonechats.**

| | **song rate** | **element rate** | **peak frequency** | **maximum frequency** | **minimum frequency** |
|---|---|---|---|---|---|
| **East Africa ↔ Europe**<br>intercept (East Africa) | 2.1 [1.91, 2.29] | 2.04 [1.93, 2.16] | 4300.9 [4165.4, 4422.9] | 8.69 [8.65, 8.75] | 7.8 [7.93, 7.66] |
| Europe | **0.64 [0.28, 1.01]** | **0.66 [0.42, 0.89]** | **601.7 [371.9, 856.3]** | 0.08 [- 0.009, 0.17] | 0.15 [- 0.1, 0.42] |
| **East Africa** | | | | | |
| LRT<br>mating, parental | χ2 = 309.3,<br>df = 1,<br>**p < 0.001** | χ2 = 0.01,<br>df = 1,<br>p = 0.9 | χ2 = 15.2,<br>df = 1,<br>**p < 0.001** | χ2 = 0.1,<br>df = 1,<br>p = 0.7 | χ2 = 6.3,<br>df = 1,<br>**p = 0.01** |
| LRT<br>prenesting, nestbuilding, incubation, parental | χ2 = 313.1,<br>df = 3,<br>**p < 0.001** | | χ2 = 25.9,<br>df = 3,<br>**p < 0.001** | | χ2 = 7.6,<br>df = 3,<br>*p = 0.055* |
| intercept (prenesting) | 9.7 [7.9, 11.4] | | 4139.6 [3932.6, 4352.9] | | 2472.3 [2226, 2722] |
| nestbuilding | *-1.6 [-3.8, 0.6]* | | **314.2 [102.3, 521.2]** | | 8.6e-03 [-2.4e+02, 2.5e+02] |
| incubation | -0.2 [-2.4, 2.1] | | -18.0 [-248.2, 198.1] | | -179 [-436.5, 86.2] |
| parental | 1.1 [-1.0, 3.4] | | -90.4 [-332.8, 140.1] | | -272.1 [-558.1, 32.0] |

In order to determine the effect of breeding stage on song parameters in East African stonechats, we first included breeding stage as factor with two levels only (mating, parental). If significant we repeated the analysis with breeding stage as factor with four levels (prenesting, nestbuilding, incubation, parental). Other rows show Bayesian estimates and credible intervals. When zero is not included within the credible intervals there is a 'significant' effect of this parameter on the dependent variable. Estimates of cofactors refer to differences from the intercept estimate. 'Significant' differences are shown in bold.

## Behavioural response to simulated territorial intrusions

Latency to attack the decoy and the overall strength of the response to STIs did not depend on latitude (attack latency: LRT: $\chi2 = 0.04$, df = 1, p = 0.8, table 5.4, fig. 5.6; behavioural response score: LRT: $\chi2 = 0.02$, df = 1, p = 0.9, table 5.4). However, the response to STI changed with breeding stage. Males attacked the intruder faster during nestbuilding and the parental stages than during prenesting (LRT: $\chi2 = 14.5$, df = 3, p = 0.002, table 5.4, fig. 5.6). Equally the behavioural response score was strongest during nestbuilding and in general stronger during all breeding stages than during prenesting (LRT: $\chi2 = 33.4$, df = 3, $p < 0.001$; df = 99, nestbuilding: t = -2.7, p = 0.007; egglaying and incubation: t = -1.9, p = 0.05; parental: t = -2.37, p = 0.02, table 5.4).

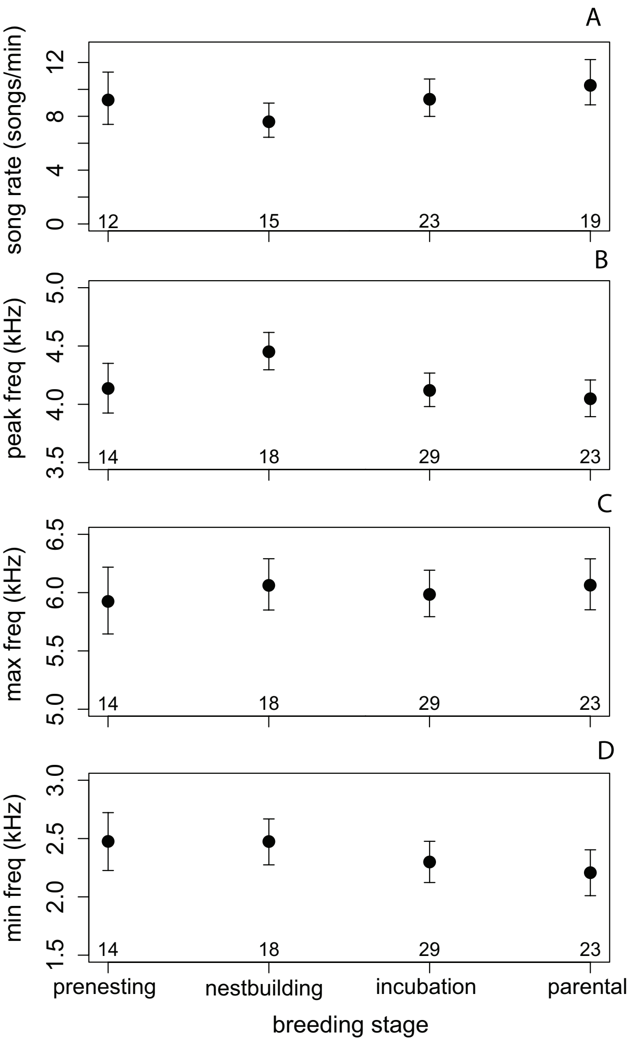

**Fig. 5.5 Dawn song in relation to breeding stage in East African stonechats with a) song rate, b) peak frequency, c) maximum frequency, and d) minimum frequency.** Males sang with a higher song rate and peak frequency during nestbuilding and with a higher minimum frequency during prenesting and nestbuilding than during the parental stages. Maximum frequency did not significantly differ between stages. Points represent posterior means and error bars 95 % credible intervals. Sample sizes are displayed below error bars.

**Table 5.4The behavioural response to STIs in relation to breeding stage.**

| | **intercept (East Africa, prenesting)** | **Europe** | **nestbuilding** | **egglaying, incubation** | **parental** |
|---|---|---|---|---|---|
| **attack latency** | | | | | |
| 2.5 % | 2.38 | - 0.39 | **- 1.5** | - 0.54 | **- 1.09** |
| 50 % | 2.76 | 0.05 | **- 0.96** | 0.04 | **- 0.59** |
| 97.5 % | 3.11 | 0.48 | **- 0.4** | 0.6 | **- 0.09** |
| | | | | | |
| **behavioral response score** | | | | | |
| 2.5 % | 0.3 | - 0.46 | **- 3.31** | **- 1.45** | **- 1.46** |
| 50 % | 0.66 | 0.02 | **- 1.92** | **- 0. 74** | **- 0.82** |
| 97.5 % | 1.00 | 0.54 | **- 0.54** | **- 0.00** | **- 0.17** |

Rows show Bayesian estimates and credible intervals. When zero is not included within the credible intervals there is a ‘significant’ effect of this parameter on the dependent variable. Estimates of cofactors refer to differences from the intercept estimate. ‘Significant’ differences are shown in bold.

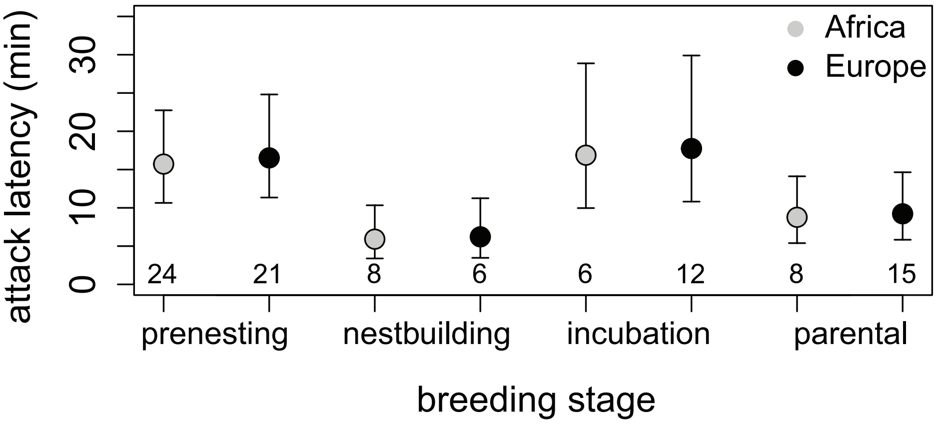

**Fig. 5.6 Latency to attack of East African and European stonechats in response to simulated territorial intrusions during different stages of the breeding season**. Male stonechats attacked the decoy faster during nestbuilding and parental stages than during prenesting. Points represent posterior means and error bars 95 % credible intervals. Sample sizes are displayed below error bars.

# Discussion

The pace of life hypothesis predicts that tropical species invest less into reproduction, have lower testosterone levels and a lower mating effort than temperate species (Ricklefs and Wikelski 2002; Hau et al. 2010). In accordance with previous studies, we found that stonechats from various East African populations had smaller clutches than stonechats from European populations (Gwinner et al. 1995a). In addition, while in East Africa the majority of males caught were adults; most males were in their first year of life in the European populations. Our data on local apparent survival fit well with earlier studies where a mortality rate of 59 % was found in a German population (Flinks and Pfeifer 1984), but of only 24 – 35 % in a Tanzanian population (Scheuerlein 2000). Thus, East African tropical stonechats indeed follow a slower pace of life than temperate European stonechats. However, despite a lower reproductive output per year, East African males had equally high testosterone levels during breeding as European stonechats with a peak during nestbuilding in all populations. Furthermore, East African stonechats responded as aggressively to an intruder as European stonechats and in all populations the behavioural response to STIs peaked during nestbuilding. In contrast, dawn song was more intense in European stonechats as they sang with a higher song rate, element rate and peak frequency during the mating period (prenesting and nestbuilding) than East African stonechats.

## Testosterone and pace of life

Contrary to predictions with respect to pace of life and previous studies in other tropical biomes (Hau et al. 2010), quantitative differences in testosterone levels in stonechats do not mediate pace of life: stonechats from East Africa with a slow pace of life did not have lower testosterone levels than those from Europe with a fast pace of life. Further, in East African and European stonechats testosterone levels peaked during nestbuilding and were low during the parental stages similar to a previous study in this species (Goymann et al. 2006). This discrepancy with other studies in which lower breeding testosterone levels were found in tropical birds can be explained with methodological differences between studies and / or ecological differences between species. Stonechats are socially monogamous seasonal breeders with a relatively short defined breeding season in Europe as well as in East Africa while the majority of studies so far have focused on neotropical or Australian species with extended breeding seasons or cooperative breeding systems (Levin and Wingfield 1992; Wikelski et al. 1999, 2003a; Peters et al. 2001; Wiley and Goldizen 2003; Fedy and Stutchbury 2006; Gill et al. 2008; Hau et al. 2010). Long breeding seasons can lead to low breeding synchrony and low competition between pairs (Wikelski et al. 1999), potentially making high testosterone levels unnecessary. Few other studies in seasonal tropical species indicate that high testosterone levels in tropical birds might not be uncommon (Moore et al. 2004; Goymann et al. 2006). For example, an equatorial population of rufous-collared sparrows *(Zonotrichia capensis)* elevated testosterone levels even more in response to a physiological challenge with gonadotropin releasing hormone during breeding than their temperate congeners (Moore et al. 2002). On the other hand, some studies in tropical birds might have missed a short peak in testosterone during the fertile period of the female. In populations of tropical birds with extended breeding seasons individual pairs can be found in any specific breeding stage at any time during the breeding season (Class and Moore 2011). Many studies, however, did only assess if males were in breeding condition or not, without further information on the exact breeding stage of individuals (Wikelski et al. 1999; Hau et al. 2010; Addis et al. 2013). Thus, any peaks in testosterone levels may be averaged out and it may be – mistakenly - concluded that these tropical birds show low testosterone levels during the whole breeding season. This may also have been the case in the two previous studies on tropical stonechats that found low year-round testosterone levels (Dittami and Gwinner 1985; Rödl et al. 2004). In these two studies testosterone levels were regressed against month of the year. In addition one of these studies was in captive birds that were not allowed to breed (Rödl et al. 2004). Furthermore, if nestbuilding and egglaying are combined as one breeding stage („the fertile period“), as is often the case, peak testosterone levels and, thus, the change from prenesting to nesting, may be underestimated even in temperate species. For example, in previous studies on

European stonechats, the peak in testosterone during nestbuilding did not reach significance. Schwabl et al combined the nestbuilding and the egglaying phase in their analysis and, thereby, may have diluted a peak in testosterone during nestbuilding.

## Territorial behaviour and pace of life

Song is one of the most important sexually selected traits in birds and important for territory establishment and mate attraction (Otter and Ratcliffe 1993). Previous studies have shown that song is under strong sexual selection (Mortega et al. 2014) and may be used to communicate the males' quality in a reproductive context in stonechats (Greig-Smith 1982b). In our study, European stonechats sang with a higher song rate, element rate and peak frequency during mating than East African stonechats. While East African stonechats are resident and stay with their partners year-round (Dittami and Gwinner 1985), European stonechats are migratory and have to establish a territory and attract prospecting females upon arrival on their breeding grounds (Roth et al. 2009). Furthermore, in Europe most males return as yearlings, thus they have to establish a territory and form a pair bond "from scratch". In many species male song rate peaks during the fertile period of their females (Bruni and Foote 2014) and a high element rate has been described in courtship songs of several species. Furthermore, higher song frequencies do not propagate through space as far as lower frequencies, and thus can be used for short-range signalling within close proximity to fertile females (Titus 1998). A high song activity and peak frequency have been associated with courtship displays and have been proposed to elicit female preference and fertility stimulation (Titus 1998; Gahr et al. 2001). Thus, a high song and element rate and a high peak frequency may help the fast acquisition of a mate and promote early laying which is crucial for breeding success in many species (Velmala et al. 2015). Therefore, migratory populations are under stronger sexual selection and have to invest more into sexually selected traits such as song than resident populations (Collins et al. 2009). Thus, differences in song activity probably reflect a resident life style of East African compared to European stonechats. As residency is common in tropical species (Newton 2007), it may have contributed to an in general slower pace of life in the tropics.

In contrast, the response to STIs did not differ between East African and European stonechats. Male stonechats from all latitudes vigorously attacked a simulated intruder throughout the breeding season. They attacked fastest during nestbuilding and the parental phase which indicates two major functions of territorial behaviour in stonechats: to guard their fertile female from other males and to defend food resources especially needed when they have to feed young. Although tropical stonechats have a much smaller clutch than temperate stonechats, they invest a high amount of time into their offspring by taking care of their fledglings for much longer than their temperate relatives similar to other tropical songbirds (Russell et al. 2004). This long investment may render mate guarding and the prevention of extrapair fertilizations important to tropical stonechats. Extrapair paternity is much less well studied in tropical compared to temperate birds, but there seems to be huge variation between species depending on breeding synchrony between pairs (Stutchbury et al. 2007; Stutchbury and Morton 2008; Eikenaar et al. 2013; Bonier et al. 2014). As stonechats breed seasonally and relatively synchronous, extrapair copulations may pose a high risk to males during the fertile period of their mates at all latitudes. Thus, intense mating guarding during nestbuilding is probably equally important for both tropical and temperate stonechats to prevent extrapair paternity and is not related to a fast pace of life of temperate stonechats.

## Testosterone and territorial behaviour

In many species testosterone mediates territorial behaviour during breeding (Wingfield et al. 1990). Experimental elevation of testosterone can result, for example, in larger territories (Chandler et al. 1994) or increased aggression during territorial fights (Beletsky et al. 2010). In migratory European stonechats the intensity of territorial aggression is regulated by testosterone during the breeding season: treatment of males with androgen and estrogen inhibitors almost doubled the latency to attack during STIs (Canoine and Gwinner 2002; Marasco et al. 2011). Likewise, similar treatment with inhibitors during the egglaying stage reduced the likelihood of dawn song in male great tits *(Parus major,* (Van Duyse et al. 2005)). Thus, while breeding season territorial aggression and dawn song is activated by testosterone, our study suggests that the fine-tuning of territorial behaviour and dawn song between different breeding stages only partly depends on testosterone in tropical and temperate stonechats (see also (Schwabl et al. 2005)): testosterone was elevated during prenesting, peaked during nestbuilding and was low during all other breeding stages, while the behavioural response to STIs was highest during nestbuilding and the parental stages of the breeding cycle. In addition, in the East African stonechats dawn song rate was lowest during nestbuilding when testosterone levels were highest. Furthermore, while in some species territorial fights led to a short-term increase in testosterone levels in the rivals (Wingfield and Wada 1989), testosterone levels did not increase in response to a simulated territorial intruder in a temperate German population. It is still not clear while in some species testosterone and territorial behaviour are tightly reciprocally linked and in others not (Goymann 2009). It has been suggested that in species that defend territories also outside a breeding context testosterone may not mediate territorial behaviour or may be only important for certain aspects of the territorial response (Goymann and Landys 2011; Apfelbeck et al. 2013a,b). Instead, testosterone seems to be associated with female receptivity in these species (Wiley and Goldizen 2003; Goymann and Landys 2011) and mediate mate guarding (van de Crommenacker et al. 2004). Recently it has been suggested that year-round territorial birds should show a maximum in testosterone during the fertile period of the female, while birds that establish new territories at the beginning of the breeding season should show a maximum during prenesting irrespective of a tropical or a temperate breeding environment (Goymann and Landys 2011). Tropical resident stonechats are territorial year-round (Dittami and Gwinner 1985) and temperate migratory stonechats defend territories on their wintering grounds (Gwinner et al. 1994). Thus, territoriality in stonechats is not tightly linked with reproduction. Accordingly, East African and European stonechats showed a peak in testosterone levels during nestbuilding when their females were most fertile and not at the beginning of the breeding season when territories were (re)-established. Surprisingly, testosterone levels were only elevated during nestbuilding and declined immediately with the start of egglaying. Although female songbirds are fertile during nestbuilding and egglaying, in most species copulation frequency is highest a few days before the start of egglaying and drops sharply with the first egg being laid (Birkhead and Moller 1993). Thus, in seasonally breeding socially monogamous species males should closely guard their females during nestbuilding to prevent extrapair copulations. Stonechat males can often be observed in close vicinity of their females early in the breeding season and copulation usually takes place 1-4 days before the first egg is laid (Urquhart 2010). Furthermore, in our study male stonechats were most aggressive towards STIs during the nestbuilding stage. Further, East African stonechats sang with a lower song rate during the nestbuilding stage. This may seem counterintuitive at first glance as in many species dawn song increases when the female is fertile (Mace 1987). Similar to stonechats male black-capped chickadees *(Poecile atricapillus)* sang least during the dawn chorus when their mate was receptive (Foote et al. 2008). In addition, they reduced their movements and stayed in the vicinity of the nest; probably in an attempt to prevent extra pair copulations of their female (Foote et al. 2008). In our study, East African stonechats not only sang less, but they also sang with a higher peak frequency than during other breeding stages, which is a characteristic of low-range song that is directed at females close by and used to avoid eavesdropping by neighbouring males (Marten and Marler 1977; Marten et al. 1977; Dabelsteen et al. 1998). In the same vein, polygynous dark-eyed juncos *(Junco hyemalis)* implanted with testosterone increased space use of males during incubation (Beletsky et al. 2010), but testosterone had no effect during the fertile period of their female (Chandler et al. 1997). We were unable to obtain detailed information on song activity across the breeding season in European stonechats. However, in an earlier study - in contrast to what we found in East African stonechats - temperate stonechats sang with a higher song rate just before egglaying (Greig-Smith 1982a). This fits well, however, with the idea that temperate stonechats experience stronger sexual selection and have to invest more into mate attraction than tropical stonechats. While both species guard their females during the fertile period, this is more pronounced in East African stonechats. We suggest that testosterone activates breeding territoriality in stonechats and that the peak in testosterone during nestbuilding facilitates mate guarding behaviour both in tropical and in temperate male stonechats. In tropical stonechats this may also include changes in song behaviour (e.g. lower song activity), while in European stonechats the need to attract and keep a mate may favour a high song activity during prenesting and the fertile period of females. We, therefore, expect that East African stonechats may have lower levels of extrapair paternity than European stonechats, in which around 21 % of nests contain extrapair young (unpublished data). Changes in territorial behaviour later in the breeding season seem to be mediated by other factors than testosterone, probably to avoid negative effects of elevated testosterone on parental behaviour (Lynn and Wingfield 2008).

## Conclusions

This is the first study on the pace of life that assessed the role of testosterone in relation to territorial behaviour in several closely related subspecies of a songbird that breeds at different latitudes from Europe to the Afrotropics. Although migratory European stonechats have to invest more into song as a sexually selected trait than resident East African stonechats, this is not associated with higher testosterone levels in European compared to East African stonechats. Thus, in male stonechats – a seasonal breeder in tropical and temperate environments - testosterone seems to play a similar role during breeding at all latitudes and does not mediate the trade-off between reproduction and survival on a between species level. As the peak in testosterone is limited to a short period of time, potential benefits of high testosterone levels may outweigh the costs of testosterone and may not have a major impact on male survival. Later stages of the breeding cycle, i.e. taking care of nestlings and fledglings, may have a greater influence on the trade-off between reproduction and survival in males of socially monogamous species. A smaller clutch greatly reduces the workload of nestling feeding and many tropical songbirds only have a single clutch during each breeding season. Future studies should focus on these later breeding stages and physiological mechanisms that mediate parental investment, e.g. corticosterone and progesterone (Hau et al. 2010).

# Acknowledgements

We thank Michaela Hau for valuable discussions about the study, Ann Gathitu, Ireene Madindou, Soi Bernard, Wilberforce Okeka, and David Ndege for assisting in the field, and Monika Trappschuh for analyzing the testosterone samples. We also thank the Museums of Kenya (NMK) and the Tanzania Wildlife Research Institute (TAWIRI) for providing support for field work in Kenya and Tanzania. BA received a Alexander von Humboldt Research Fellowship. K.G.M. was supported by the International Max Planck Research School for Organismal Biology. The study was funded by the German Science foundation (research grant to BH and KGM). The funders had no role in study design, data collection and analysis or preparation of the manuscript.

# PART 3: Evolutionary implications of divergence in genes, song, and seasonality

# CHAPTER 6 Characterization of microsatellite markers for *Saxicola* species

*Mortega KG, Horsburgh GJ, Illera JC, Dawson DA. 2015 Conservation Genet Resour 7:273–278*

# Abstract

We characterized 28 unique African stonechat (*Saxicola torquata*) microsatellite loci. Seventeen loci characterized in 24 unrelated *Saxicola torquata axillaris* individuals sampled at Mount Meru, Tanzania displayed 2– 26 alleles per locus and observed heterozygosities ranged from 0.29 to 0.92. Heterozygous females and sequence similarity suggested all 17 loci were autosomal. All markers also successfully amplified in nine different species ranging from Europe to Asia, including three endemic Island species. These microsatellite markers will be useful to assess the genetic diversity of the large and widely distributed genus *Saxicola*, a group comprising 11 recognized species with evidences of cryptic diversification. Several species show a narrow range distribution and are of conservation concern.

# Methods and Results

Here, we present a new set of microsatellite markers isolated from a songbird, the African stonechat (*Saxicola torquata axillaris*), which will be useful to assess genetic diversity for conservation efforts in other *Saxicola* species. An Illumina paired-end library was created using 1 µg of genomic DNA extracted from a female *S. t. axillaris* (AR02F) by following the standard protocol of the SureSelect Library Prep Kit, ILM (Agilent Technologies Inc. Santa Clara, California). DNA sequencing was conducted using a MiSeq Benchtop Sequencer (Illumina Inc., San Diego, California).

Primer sets were designed for 28 unique microsatellite sequences (EMBL accession numbers: HG798924–HG798951) using PRIMER3 v0.4.0 (Rozen and Skaletsky 2000). Blood samples were obtained from 24 unrelated adult *S. t. axillaris* individuals sampled at Mount Meru, Arusha National Park, Tanzania. Genomic DNA was extracted using an ammonium acetate precipitation method.
Primer sets were amplified individually in all 24 individuals. Each 2-µl PCR contained approximately 10 ng of air-dried genomic DNA, 0.2 µM of each primer and 1 µl QIAGEN PCR mix (QIAGEN Inc.). PCR amplification was performed using a DNA Engine Tetrad ®Thermal Cycler (MJ Research, Bio-Rad, Hemel Hempstead, Herts, UK) with the following program: 95 °C for 15 min, followed by 35 cycles of 94 °C for 30 s, 58 °C for 90 s, 72 °C for 60 s, 45 cycles of 60 °C for 30 min. Locus-specific products were loaded separately on an ABI 3730 48-well capillary DNA Analyzer (Applied Biosystems, California, USA) and allele sizes assigned using GENEMAPPER v4.1 (Applied Biosystems).

**Table 6.1 Microsatellite loci for the *Saxicola* species**

a) Primer sequences and characteristics of 21 autosomal African stonechat (Saxicola torquata axillaris) microsatellite loci

| Locus | EMBL Accession number and clone name | Repeat motif | Primer sequence (5′-3′) | $T_m$ (°C) | n | Exp. allele size (bp) | Obs. allele size range and genotype of AR02F$^{¥}$ (bp) | $N_A$ | $H_E$ | $H_O$ | $_p$HWE | Est. null allele freq. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Stor01 | HG798924 | (ATCC)$_{16}$ | F:[HEX]CTCATCCTCTGCTTCCATCTG | 59.96 | 24 | 194 | 181–209 | 19 | 0.81 | 0.85 | 0.63 | 0.02 |
|  | STC10640 |  | R:GATCAACTGATGTCATCCATGC | 60.36 |  |  | 193, 205 |  |  |  |  |  |
| Stor02 | HG798925 | (GT)$_{10}$ | F:[6FAM]TGGGCTCAAATTGAACTGTG | 59.69 | 23 | 198 | 194–202 | 6 | 0.64 | 0.63 | 0.14 | 0.01 |
|  | STC10944 |  | R:CTTTGTGCTGCTGCTTTCAC | 59.79 |  |  | 198 |  |  |  |  |  |
| Stor03 | HG798926 | (ATCC)$_{13}$ | F:[HEX]AACTGGAGGTCCAGGCATC | 60.06 | 22 | 161 | 159–183 | 15 | 0.92 | 0.84 | 1.00 | -0.05 |
|  | STC12196 |  | R:GAGTGGCCTGTGTGTGGAG | 60.31 |  |  | 159, 171 |  |  |  |  |  |
| Stor04 | HG798927 | (ATGTT)$_{21}$ | F:[HEX]TCCTAAATGCGACATTGCAC | 59.69 | 24 | 199 | 151–207 | 19 | 0.85 | 0.89 | 0.05 | 0.02 |
|  | STC13188 |  | R:GCAAGGTAAATTTGTCTTCTTGTG | 59.27 |  |  | 191, 197 |  |  |  |  |  |
| Stor05 | HG798928 | (AC)$_{13}$ | F:[HEX]GCCCACTCAGCTGACACAC | 60.48 | 21 | 199 | 191–197 | 12 | 0.29 | 0.27 | 1.00 | -0.07 |
|  | STC13366 |  | R:TGTGTTGTGCATCATAGTGTATGG | 60.73 |  |  | 195 |  |  |  |  |  |
| Stor06 | HG798929 | (AC)$_{15}$ | F:[6FAM]CTTGTTGCTCACCCTGTGG | 60.29 | 21 | 156 | 145–159 | 14 | 0.71 | 0.65 | 0.80 | -0.05 |
|  | STC15546 |  | R:TAGAGGCAGCCAACCTTCTG | 60.53 |  |  | 151, 153 |  |  |  |  |  |
| Stor07 | HG798930 | (GT)$_{15}$ | F:[6FAM]CTGTCTGGGCATGAGAAGG | 59.35 | 24 | 153 | 139–153 | 8 | 0.65 | 0.65 | 0.28 | -0.03 |
|  | STC18373 |  | R:TTTGCAGTCAGTCAGTACAAAGC | 59.62 |  |  | 143, 153 |  |  |  |  |  |
| Stor08 | HG798931 | (ATCC)$_{18}$ | F:[6-FAM]CACAGCTGCTCTGGGAATC | 59.52 | 24 | 162 | 220–236 | 9 | 0.50 | 0.38 | 0.27 | -0.14 |
|  | STC2321 |  | R:ACAAGGGATGGAGGGACAG | 59.90 |  |  | 224 |  |  |  |  |  |
| Stor09 | HG798932 | (GT)$_{13}$ | F:[HEX]TCGGTGTCTGTTGGTATTGC | 59.57 | 19 | 200 | 200–224 | 8 | 0.74 | 0.77 | 0.69 | 0.01 |
|  | STC2764 |  | R:GCAGCTGCCTTTCTGTATGTC | 60.04 |  |  | 202, 208 |  |  |  |  |  |
| Stor10 | HG798933 | (TAA)$_{13}$ | F:[6FAM]TTGAAAGGTTACCCTGTTTGTG | 59.04 | 23 | 210 | 195–221 | 26 | 0.89 | 0.85 | 0.46 | -0.03 |
|  | STC3033 |  | R:GGTACATTTCTGCTTTCAGATCC | 59.15 |  |  | 207, 213 |  |  |  |  |  |
| Stor11 | HG798934 | (GT)$_{15}$ | F:[HEX]AGAGTGGCAACTTGTTCTTGG | 59.39 | 24 | 143 | 139–147 | 15 | 0.71 | 0.67 | 0.84 | -0.04 |

| Locus | EMBL Accession number and clone name | Repeat motif | Primer sequence (5′-3′) | $T_m$ (°C) | n | Exp. allele size (bp) | Obs. allele size range and genotype of AR02F$^{¥}$ (bp) | $N_A$ | $H_E$ | $H_O$ | $_{P}$HWE | Est. null allele freq. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | STC3205 | | R:TGTACCAGCTCTGCATCATTG | 59.88 | | | 139, 143 | | | | | |
| Stor12 | HG798935 | (TGA)$_{18}$ | F:[HEX]GGCAGTGGCATCTGATTTG | 60.22 | 24 | 217 | 193–239 | 23 | 0.35 | 0.29 | 0.56 | -0.09 |
| | STC3964 | | R:GGGAGATTCTCAACAGTGCAG | 59.86 | | | 193 | | | | | |
| Stor13 | HG798936 | (TGA)$_{19}$ | F:[HEX]TGCTGACAAAGGGAGAAAGC | 60.52 | 23 | 214 | 189–215 | 25 | 0.86 | 0.86 | 0.12 | -0.01 |
| | STC4021 | | R:CTGTAATCCGGCAGCACAC | 60.28 | | | 193, 205 | | | | | |
| Stor14 | HG798937 | (GT)$_{12}$ | F:[6-FAM]CCATGTAAAGCACTTCCAAACC | 60.75 | 22 | 141 | 138–144 | 8 | 0.75 | 0.75 | 0.42 | -0.01 |
| | STC4937 | | R:GGGAGAGGCAGGAACTGG | 60.76 | | | 142 | | | | | |
| Stor15 | HG798938 | (CT)$_{11}$ | F:[6FAM]TCATTAAGGTTTGACTGTGTTGC | 59.19 | 21 | 125 | 120–124 | 7 | 0.46 | 0.49 | 0.05 | 0.06 |
| | STC7592 | | R:AAGGGCAAGATTTCCTGTTG | 59.17 | | | 120, 124 | | | | | |
| Stor16 | HG798939 | (AC)$_{15}$ | F:[6FAM]AAACAGAACAAACCTCCATGTG | 59.00 | 23 | 201 | 194–206 | 5 | 0.60 | 0.64 | 0.65 | 0.02 |
| | STC11720 | | R:CTCCAGCCTATTGTATCATCACTATC | 59.49 | | | 200 | | | | | |
| Stor17 | HG798940 | (CAAA)$_{13}$ | F:[HEX]AACAGAACTGCTGGCAACTG | 59.09 | 22 | 232 | 221–237 | 5 | 0.70 | 0.76 | 0.15 | 0.02 |
| | STC2521 | | R:TTGATGGTTTCAGCATGGTC | 59.50 | | | 233 | | | | | |
| Stor18$^{*}$ | HG798941 | (CTG)$_{11}$ | F:[HEX]GAGTCTCCAAGCTCTGCCTTC | 60.66 | 24 | 155 | 145–170 | 7 | 0.69 | 0.71 | **0.02** | 0.02 |
| | STC10500 | | R:TCCTTGAGGAGAGGGTTAAAGG | 60.93 | | | – | | | | | |
| Stor23$^{*}$ | HG798946 | (GGAT)$_{16}$ | F:[HEX]GCATCCTGAGACCATGTGTG | 60.12 | 24 | 193 | 191–215 | 8 | 0.83 | 0.79 | **0.01** | -0.03 |
| | STC2689 | | R:CGTCCATACCCATGTCAGTG | 59.83 | | | 195, 199 | | | | | |
| Stor24$^{*}$ | HG798947 | (ATCC)$_{22}$ | F:[6FAM]AGAGCTGAGTCCTTCCCAAAG | 60.00 | 23 | 240 | 238–256 | 5 | 0.48 | 0.90 | **0.00** | 0.30 |
| | STC4625 | | R:CCTGGATCAGGCAAGTGG | 60.20 | | | – | | | | | |
| Stor26$^{*}$ | HG798949 | (ATCC)$_{12}$ | F:[HEX]AATTCTTCATCCCATTTCCATTATAG | 59.86 | 23 | 152 | 137–159 | 7 | 0.79 | 0.76 | **0.00** | 0.11 |
| | STC6257 | | R:GAGCTGGGACCACAAGATTC | 59.66 | | | – | | | | | |

b) Cross amplification of 16 *Saxicola torquata axillaris* microsatellite loci for ten *Saxicola* species

| | *S.t. axillaris* Kenya (24 tested) | | | *S.t. rubicola* Spain (30 tested) | | | | | *S. caprata* Indonesia (7 tested) | | *S. dacotiae* Canary Islands (24 tested) | | | | | *S. gutturalis* Timor-Leste (6 tested) | | *S. insignis* Nepal (1 tested) | *S. maura* Kazakhstan (4 tested) | | *S. rubetra* Spain (15 tested) | | | | | *S. sibilla* Madagascar (6 tested) | | *S. tectes* Réunion (1 tested) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Locus | Exp. allele size (bp) ¥ | n | Obs. allele size range and genotype of AR02F¥ (bp) | n | Obs. allele size range (bp) | $H_E$ | $H_O$ | $_P$HWE | n | Obs. allele size range (bp) | n | Obs. allele size range (bp) | $H_E$ | $H_O$ | $_P$HWE | n | Obs. allele size range (bp) | Obs. allele size range (bp) | n | Obs. allele size range (bp) | n | Obs. allele size range (bp) | $H_E$ | $H_O$ | $_P$HWE | n | Obs. allele size range (bp) | Obs. allele size range (bp) |
| Stor01 | 194 | 24 | 181–209<br>193, 205 | 24 | 160–230 | 0.81 | 0.83 | **0.03**$^{*}$ | 6 | 182–226 | 19 | 182–202 | 0.63 | 0.74 | 1.00 | 3 | 172–226 | - | 1 | 198–226 | 4 | 160–226 | 0.86 | 0.75 | 0.64 | 4 | 190–218 | 206–21 |
| Stor02 | 198 | 23 | 194–202<br>198 | 22 | 186–206 | 0.48 | 0.46 | **0.007**$^{*}$ | 7 | 190–204 | 21 | 192–198 | 0.18 | 0.14 | 0.14 | 5 | 190–204 | 194–196 | 2 | 192–196 | 8 | 190–202 | 0.77 | 0.50 | 0.05 | 6 | 198–202 | 200–202 |
| Stor03 | 161 | 22 | 159–183<br>159, 171 | 26 | 155–177 | 0.73 | 0.73 | 0.21 | 6 | 159–179 | 19 | 155–177 | 0.82 | 0.90 | 0.90 | 6 | 147–175 | 155–163 | 2 | 163 | 11 | 147–187 | 0.87 | 0.55 | **0.03**$^{*}$ | 6 | 163–219 | 183–191 |
| Stor04 | 199 | 24 | 151–207<br>191, 197 | 21 | 133–201 | 0.88 | 0.86 | 0.92 | 6 | 133–191 | 20 | 137–187 | 0.87 | 0.65 | **0.03**$^{*}$ | 3 | 147–207 | 137–145 | 2 | 149–161 | 12 | 131–159 | 0.88 | 0.75 | **0.005**$^{*}$ | 6 | 151–217 | 193 |
| Stor05 | 199 | 21 | 191–197<br>195 | 27 | 181–197 | 0.14 | 0.11 | 0.11 | 6 | 189–195 | 22 | 187–193 | 0.31 | 0.36 | 1.00 | 6 | 187–195 | 191–193 | 3 | 189–191 | 14 | 177–203 | 0.70 | 0.71 | 0.40 | 6 | 195–199 | 195 |
| Stor06 | 156 | 21 | 145–159<br>151, 153 | 26 | 147–155 | 0.55 | 0.54 | 0.05 | 7 | 139–153 | 22 | 149–153 | 0.53 | 0.59 | 0.84 | 5 | 143–159 | - | 3 | 149–151 | 13 | 139–153 | 0.89 | 0.85 | 0.68 | 6 | 145–155 | 151–153 |
| Stor07 | 153 | 24 | 139–153<br>143, 153 | 27 | 150–158 | 0.67 | 0. | 0.14 | 7 | 144–174 | 22 | 148–150 | 0.30 | 0.36 | 1.00 | 6 | 128–158 | 150 | 3 | 146–156 | 12 | 144–204 | 0.91 | 0.67 | **0.002**$^{*}$ | 6 | 144–166 | 150–152 |
| Stor08 | 162 | 24 | 220–236<br>224 | 26 | 224 | - | - | - | 5 | 224 | 20 | 224 | - | - | - | 4 | 224 | 224 | 2 | 224 | 11 | 224 | - | - | - | 6 | 224 | 224 |
| Stor09 | 200 | 19 | 200–224<br>202, 208 | 25 | 200–236 | 0.88 | 0.88 | 1.00 | 5 | 202–230 | 20 | 190–200 | 0.31 | 0.20 | **0.01**$^{*}$ | 4 | 196–218 | - | 2 | 190–202 | 11 | 196–220 | 0.84 | 0.46 | **0.0003**$^{*}$ | 5 | 200–214 | 204–214 |

| Locus | *S.t. axillaris* Kenya (24 tested) | | | *S.t. rubicola* Spain (30 tested) | | | | | *S. caprata* Indonesia (7 tested) | | *S. dacotiae* Canary Islands (24 tested) | | | | | *S. gutturalis* Timor-Leste (6 tested) | | *S. insignis* Nepal (1 tested) | *S. maura* Kazakhstan (4 tested) | | *S. rubetra* Spain (15 tested) | | | | | *S. sibilla* Madagascar (6 tested) | | *S. tectes* Réunion (1 tested) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Exp. allele size (bp) ¥ | n | Obs. allele size range and genotype of AR02F¥ (bp) | n | Obs. allele size range (bp) | $H_E$ | $H_O$ | $_{\mathrm{P}}$HWE | n | Obs. allele size range (bp) | n | Obs. allele size range (bp) | $H_E$ | $H_O$ | $_{\mathrm{P}}$HWE | n | Obs. allele size range (bp) | Obs. allele size range (bp) | n | Obs. allele size range (bp) | n | Obs. allele size range (bp) | $H_E$ | $H_O$ | $_{\mathrm{P}}$HWE | n | Obs. allele size range (bp) | Obs. allele size range (bp) |
| Stor10 | 210 | 23 | 195–221<br>207, 213 | 25 | 190–194 | 0.18 | 0.12 | 0.20 | 6 | 186–190 | 20 | 190 | - | - | - | 5 | 178–192 | 186–190 | 2 | 190–194 | 11 | 172–196 | 0.75 | 0.72 | 1.00 | 4 | 190–210 | 190–196 |
| Stor11 | 143 | 24 | 139–147<br>139, 143 | 26 | 139–173 | 0.87 | 0.65 | **0.005*** | 7 | 133–159 | 20 | 133–163 | 0.05 | 0.05 | - | 3 | 133–163 | 133–163 | 4 | 143–163 | 14 | 133–167 | 0.73 | 0.71 | 0.72 | 6 | 137–159 | 141–145 |
| Stor12 | 217 | 24 | 193–239<br>193 | 27 | 181–211 | 0.87 | 0.81 | 0.37 | 7 | 177–205 | 22 | 181–191 | 0.09 | 0.09 | 1.00 | 6 | 181–207 | 181–187 | 3 | 181–191 | 14 | 177–209 | 0.87 | 0.71 | **0.03*** | 6 | 191–235 | 205–209 |
| Stor13 | 214 | 23 | 189–215<br>193, 205 | 24 | 184–214 | 0.88 | 0.75 | **0.01*** | 6 | 166–224 | 20 | 190–198 | 0.23 | 0.25 | 1.00 | 5 | 192–208 | 182–198 | 2 | 194–208 | 9 | 176–230 | 0.92 | 0.89 | 0.63 | 3 | 188–220 | 194–196 |
| Stor14 | 141 | 22 | 138–144<br>142 | 25 | 137–159 | 0.50 | 0.48 | 1.00 | 6 | 135–159 | 18 | 137–143 | 0.29 | 0.33 | 1.00 | 4 | 153 | 137–143 | 2 | 155–163 | 10 | 137–159 | 0.92 | 0.90 | 0.77 | 6 | 137 -141 | 137 |
| Stor15 | 125 | 21 | 120–124<br>120, 124 | 28 | 121–135 | 0.57 | 0.46 | **0.001*** | 7 | 123–167 | 22 | 117–129 | 0.50 | 0.41 | 0.60 | 5 | 129–159 | 117–129 | 3 | 121–137 | 12 | 119–177 | 0.97 | 0.95 | 1.00 | 6 | 119–121 | 121 |
| Stor16 | 201 | 23 | 194–206<br>200 | 27 | 194–202 | 0.58 | 0.52 | 0.08 | 7 | 182–204 | 21 | 194–196 | 0.05 | 0.05 | - | 6 | 182–228 | 196–198 | 4 | 198–210 | 13 | 176–209 | 0.89 | 0.81 | 1.00 | 5 | 196–204 | 196–200 |

S. t. axillaris, Saxicola torquata axillaris; S. t. rubicola, Saxicola torquata rubicola; ¥ based on the sequenced Saxicola torquata axillaris individual from which primers were designed (AR02F); Tm melting temperature (ºC) of primer; bp base pairs; Exp. expected; Obs. observed allele size; NA number of alleles observed; n number of individuals genotyped, sex based on adult plumage; HO observed heterozygosity; HE expected heterozygosity; PHWE probability of deviation from Hardy-Weinberg equilibrium; Est. estimated null allelic frequency.

* Loci (shown in bold) and taxa, which deviated from Hardy-Weinberg equilibrium ($p < 0.05$): Stor18, Stor23, Stor24, and Stor26 or Saxicola torquata axillaris; Stor01, Stor02, Stor10, Stor13 and Stor15 for Saxicola torquata rubicola; Stor04 and Stor09 for Saxicola dacotiae; Stor03, Stor04, Stor07, Stor09 and Stor12 for Saxicola rubetra

The numbers of alleles and heterozygosities were calculated for each locus using CERVUS v3.0.6. Tests for deviation from Hardy–Weinberg equilibrium and linkage disequilibrium were conducted using GENEPOP web version 4.2. To identify sex-linked loci, 318 (76 females, 242 males) *S. t. axillaris* individuals were assessed for heterozygosity (sex based on adult plumage and the Z-002A and Z-002D sex-typing markers; (Dawson 2007)) and the chromosomal location of each locus was assigned in the zebra finch (*Taeniopygia guttata*) assembled genome by performing a BLAST search for sequence similarity (via http://www.ensembl.org/index.html), following Dawson et al. ((Dawson et al. 2006); homologous sequences possessed E-values less than E −05) and a figure created using MAPCHART v2.2.

Of the 28 markers tested, 21 were polymorphic with 2–26 alleles, two were monomorphic, and five amplified multiple non-specific products despite testing at various annealing temperatures (Table 6.1 and Online Resources 1 and 2). Loci *Stor20* and *Stor21* were monomorphic with the same allele sizes in three other populations (data not shown). None of the 21 polymorphic loci were sex-linked based on the presence of female heterozygotes and sequence similarity to zebra finch autosomes (Fig. 6.1). Locus *Stor26* displayed a high estimated null allele frequency (over 10 %). No groups of loci exhibited evidence of linkage disequilibrium.

Sixteen selected markers (*Stor01–Stor16*) were assessed in nine other *Saxicola* species, including taxa classified as near threatened (*S. dacotiae* and *S. gutturalis*) or vulnerable (*S. insignis*; Table 6.1 and Online Resource 1). Between 11 and 16 loci were variable per species (1–24 individuals tested per species). The avian genus *Saxicola* is distributed throughout Africa, Asia, Europe and various islands across Oceania (Illera et al. 2008). Many species within this complex are listed as endangered. Thus, these microsatellites will be useful to assess genetic diversity for conservation efforts.

# Acknowledgements

We thank Terry Burke and Barbara Helm for support. DNA extraction, genotyping and sex-typing were performed at the NERC Biomolecular Analysis Facility at the University of Sheffield, funded by the Natural Environment Research Council (NERC), UK. Next generation sequencing data was provided by Darren Grafham and Jennifer Dawe at Sheffield Diagnostic Genetics Services (SDGS) of the Sheffield Children's Hospital Foundation Trust (SCHfT), UK. Andy Krupa provided technical support. K.G.M. was supported by the International Max Planck Research School for Organismal Biology and the German Science Foundation.

# CHAPTER 7 Allochronic and geographic reproductive isolation in an African songbird

*Mortega KG, van Toor M, Illera JC, Apfelbeck B, Johnson PCD, Matthiopoulos J, Dawson DA, Grant BR, Burke T, Helm B. (manuscript)*

# Abstract

Ecological speciation - the divergence by local adaptation to different environments – is being studied extensively with a focus on the role of geographic isolation for population divergence. In contrast, allochronic isolation - the separation of populations by timing - remains mostly unconsidered when studying speciation. Populations might exhibit temporal barriers to gene flow because seasonal activities must be accurately fitted to local conditions to avoid mismatches with the environment, which can have severe fitness consequences. The evolution of rigid reproductive schedules, via endogenously generated biological rhythms, buffers organisms from responding to misleading, unpredictable variability of environments, but may thereby restrict gene flow. The African stonechat, *Saxicola torquata axillaris*, is a well-studied species with robust reproductive rhythms. We studied genetic, song, and morphological divergence between synchronic and allochronic breeding populations, and populations separated by geographic barriers. We modeled the spatial connectivity between populations to quantify the relative contributions of temporal and spatial isolation to population divergence. We found that allochronic and spatially isolated populations are genetically differentiated as judged from microsatellite loci and mitochondrial DNA. Allochronic, genetically distinct populations are spatially connected with no geographic or environmental barriers to gene flow, which strongly indicates population divergence as the result of allochronic isolation. The genetic structure is strongly concordant with patterns of song and morphology divergence of Stonechats. Notably, females preferred vocal and visual stimuli associated with synchronously breeding males, thus promoting reproductive barriers by behavioral isolation. Our results indicate that the evolutionary dynamics of allochronic isolation, reinforced by sexual selection especially for culturally transmitted song may be key drivers for population divergence and ultimately speciation.

# Introduction

Ecological speciation, i.e. divergence by local adaptation to different environments, has become recognized as an important mechanism of evolutionary change (Rundle and Nosil 2005; Nosil 2008; Elmer and Meyer 2010). Like other forms of speciation, it involves reduced gene flow and eventual reproductive isolation between populations. While contributions to isolation by spatial aspects of the environment (e.g., parapatry, micro-habitat structure in sympatry) are under extensive investigation (Huxley 1943; Coyne and Orr 2004), little attention is being paid to temporal aspects. However, the existing evidence indicates that allochrony, i.e. divergence driven by different mating time (Coyne and Orr 2004; Yamamoto and Sota 2009), can occur rapidly even without geographic isolation. Aligning biological processes with environmental rhythms (Durant et al. 2007) is thought to be under strong selection, which can act on the innate biological time-keeping mechanisms which in most organisms regulate reproductive timing. Studies of allochronic isolation in invertebrates have indeed linked its fast evolution to changes in biological rhythms (Schwarz et al. 2009; Fuchikawa et al. 2010). In tetrapods, we are aware of only two studies that provide robust evidence for accelerated diversification of populations by temporal isolation. Madeiran storm petrels, *Oceanodroma castro*, which breed sympatrically at different times of the year, display genetic isolation within the same colonies (Friesen et al. 2007). The second study, involving 57 New World bird species, relates allochronic speciation to different cycles in food availability associated with variation in precipitation regimes (Quintero et al. 2014). Although enticing, this study does not explicitly disentangle the relative roles of the spatial and temporal environment for allochrony, nor to they address the mechanistic basis, such as possible involvement of biological rhythms.

We address this knowledge gap using the Stonechat, *Saxicola torquata,* a taxon that is emblematic of endogenous, circannual rhythmicity (i.e., endogenous rhythmicity with a period length close to one year) (Dittami and Gwinner 1985; Gwinner and Dittami 1990). Stonechats are widespread songbirds with high genetic variation and well-known ecology and behavior (Dittami and Gwinner 1985; Illera et al. 2008; Woog et al. 2008; Mortega et al. 2015). In the wild, Stonechats display clear, and geographically distinct, reproductive rhythms, i.e. growth and regression of reproductive organs (Dittami and Gwinner 1985; Gwinner et al. 1995b; Helm 2009). In captivity, their distinct reproductive schedules diminished breeding success in crossbreeding trials and were shown to have a circannual basis (Gwinner and Dittami 1990; Gwinner et al. 1995b; Helm 2009). Circannual rhythms persisted for a decade in individuals kept under constant conditions and were also expressed by offspring that had never experienced environmental fluctuations (Sorek and Levy 2014). These genetically hard-wired timing programs could thereby provide potent evolutionary substrate for allochronic diversification. This possibility can be tested in Stonechat populations in East Africa, which show high genetic sub-structuring in a region of distinctly heterogeneous environmental seasonality (Jetz and Rahbek 2002; Illera et al. 2008; Woog et al. 2008). In East Africa, rainy seasons that are suitable for breeding recur within long-term predictable times of year, but these times differ regionally on fine spatial scales. Remarkably, captive Stonechats from East Africa showed particularly robust circannual rhythms, which could gate breeding to occur during the periodic rainy seasons instead of induction by spurious environmental variability (Dittami and Gwinner 1985; Sorek and Levy 2014). Stonechats could thus enhance their fitness if they aligned their circannual rhythms to local seasonality using reliable predictive cues. Even at the equator, such cues are provided by the annual changes in sunrise and sunset times, to which Stonechats have been shown to synchronize in appropriate phase position (Goymann et al. 2012).

While rhythms can feasibly act as barriers, movements and breeding dispersal may counteract local adaptation. Given the high avian dispersal ability, additional barriers might therefore be necessary to uphold the potential isolation by differences in local seasonality. Demonstrated behavioral barriers against gene flow include discrimination against non-local recruits. Specifically, signaling in the context of mate attraction may promote reproductive isolation through assortative mating and settlement, although heightened territory defense against local birds may lower their mating prospects (Grant and Grant 2002; Edwards et al. 2005; Price and Sol 2008; Podos 2010). In many songbirds, songs are a key component of signaling and are transmitted across generations via vocal learning, which often is possible only during the breeding season (Slabbekoorn and Smith 2002). Accordingly, song dialects, i.e. the unique repertoire of shared songs within a population, combined with female preference for a local dialect, may promote reproductive divergence (Marler and Tamura 1962; Nottebohm 1969; Baker 1975; Searcy 1992), presumably, vocal traits in conjunction with selected traits of different sensory (e.g., visual) modalities, such as plumage patterns including patches. Recent findings from European Stonechats make local divergence through fine local discrimination abilities of song and morphology a distinct possibility, and because song is expressed seasonally, we suggest that it may promote allochronic isolation (Mortega et al. 2014).

Clearly, temporal aspects of the environment that could potentially drive population divergence act in combination with spatial aspects. Disentangling the contributions of the temporal and spatial environment provides a major challenge, but the rapid development of spatially explicit modeling techniques offers new avenues to address it. We estimated generalized functional responses from spatial data (Matthiopoulos et al. 2011), and implemented these to obtain resistance distances (McRae et al. 2008) describing the spatial connectivity between populations. Many habitat features, such as open space or mountain ranges, may function as additional behavioral or ecological barriers (Harris and Reed 2002).

We accounted for these possibilities by studying twelve local populations of Stonechats from an extensive area in East Africa (**Fig. 7.1**; total sample size=512), in which Stonechats have previously been known to breed at high altitudes, and up to six months apart. Using newly developed molecular tools (Mortega et al. 2015), we quantified genetic diversity using 18 microsatellite loci (nDNA) to assess recent gene flow, and the mitochondrial cytochrome b (mtDNA) to reconstruct the deeper phylogenetic divergence. Combining nDNA and mtDNA markers has improved the power for testing phylogenetic and phylogeographic hypotheses. We quantified breeding time, spatial information, song, morphology, and genetic variation for the twelve study populations. Using information on song and morphology, we examined potentially concordant divergence in genetic, acoustic, and visual traits. We also tested acoustic and visual discrimination against conspecifics by playback of song and exposure to decoys. Tests were conducted to better understand behavioral mechanisms of reproductive isolation through sexual selection (Flinks et al. 2008; Illera et al. 2008), and to compare influences of distance, habitat features and synchronicity.

We used the data to distinguish between the following hypotheses: the null hypothesis is that geographical distance (D) between populations sufficiently determines genetic diversity, so that no further genetic substructure would be identified. This is contrasted with the alternative "Spatial Environment" ($E_s$) hypothesis of greater explanatory power by models that include resistance calculated from spatially explicit landscape features, and the "Temporal Environment" ($E_T$) hypothesis that proposes significant contributions of allochrony to population structure. Finally, we hypothesized that song may elicit stronger responses than morphological traits in both sexes because it may have diverged more rapidly, and because song is closely tied to seasonality.

Stonechat populations revealed considerable substructure and concordance in patterns of genotypic and phenotypic traits between three regions that related to allochronic and spatial features of the environment, implying that both restrict gene flow between populations.

# Results

## Population divergence based on mitochondrial DNA

The analysis of the genetic structure of the twelve Stonechat populations based on the cytochrome b sequence revealed a significant level of genetic differentiation among populations within each region (AMOVA, $\phi_{SC}$=0.20, $P < 0.0001$) and among the three regions ($\phi_{CT}$=0.18, $P < 0.0001$; Fig. 7.1B). Most variation was observed within populations (65.05%) and among regions (18.27%). The phylogenetic network shows a clear separation between the geographical regions of the Great Rift Valley and the Eastern Arc Mountains (**Fig. 7.1**B). The GRV late populations Monduli and Mount Meru were not separable from the GRV early populations (**Fig. 7.1**B). These two late breeding populations shared the most common haplotype with four early breeding populations of the GRV (Kakamega, Ol Kalou, Mount Kenya, and Kinangop). The analysis was based on 38 haplotypes among the 134 individuals (**Fig. 7.1**B). Using DnaSP, we identified 49 polymorphic sites of which 25 were informative. Haplotype diversity was highest for the Monduli population (GRV late, h=0.855), followed by Kakamega and Mount Kenya (GRV early, h=0.833 and 0.800), and lowest for Ngong Hills (GRV early, h=0.200) and Pare Mountains (EAM late, h=0.182; **Table 7.2**B). Nucleotide diversity was highest for early breeding populations Ol Kalou and Mataara (GRV early, $\pi$=0.00268 and 0.00185, respectively), followed by Monduli (GRV late, $\pi$=0.00158), Ngong Hills (GRV early, $\pi$=0.00019), Pare Mountains and Taita Hills (EAM late, $\pi$=0.00069 and 0.00086, respectively; **Table 7.2**B, **Fig. 7.1**B).

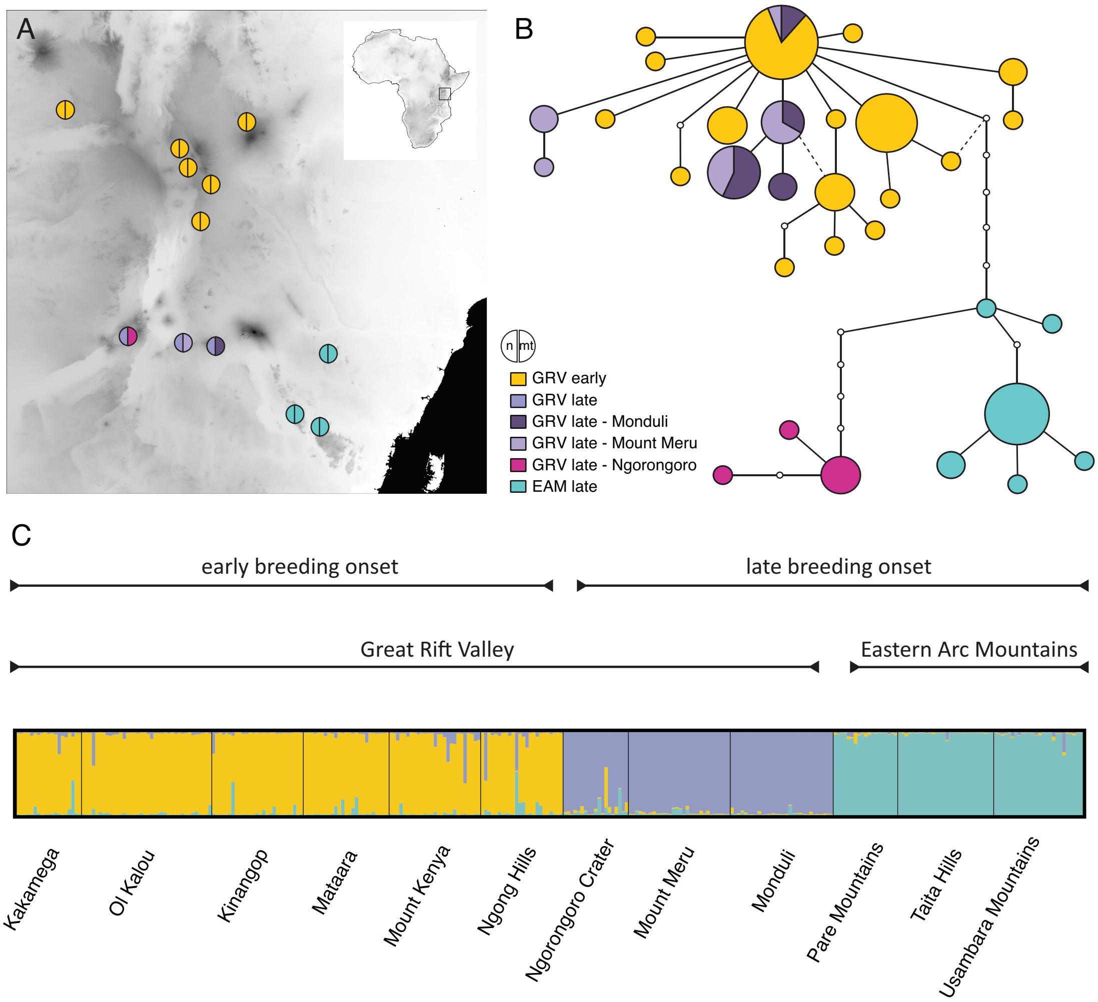

**Fig. 7.1 Population structure for 12 populations of Stonechats.** (A) Study sites color-coded according to genetic markers, nuclear (left) and mtDNA (right). (B) Minimum spanning network based on the mitochondrial cytochrome b. The sizes of haplotypes reflect the number of specimens sharing the same haplotype (N=134). (C) Bayesian population assignment test for 18 microsatellite loci with STRUCTURE uncovered three distinct clusters: i) populations from the Great Rift Valley with an early (yellow) and ii) late breeding onset (blue), and iii) populations from the Eastern Arc Mountains with a late breeding onset (green; N=312).

## Population divergence based on microsatellites

Based on 18 microsatellite loci (EMBL accession numbers: HG798924 – HG798951), the twelve Stonechat populations showed considerable genetic diversity among three geographic regions of the Great Rift Valley (GRV early and late) and the Eastern Arc Mountains (EAM late; $F_{CT}$=0.11, P<0.0001; **Table 7.1**, sample sizes are provided in **Table 7.3**; **Fig. 7.1**C). No loci were in Hardy–Weinberg or linkage disequilibrium (all P > 0.05). In general, within-site diversity was moderate as shown by the expected heterozygosity ranging from 0.54 to 0.72 (**Table 7.2**).

The two allochronic regions of the GRV, i.e. populations with an early and late breeding onset, were genetically differentiated from each other (AMOVA, $D_{EST}$=0.043 - 0.127, P<0.0001; Table 7.3), and also differed from the geographically separated populations of the second mountain range EAM ($D_{EST}$=0.122 - 0.242; **Table 7.1**, Table 7.3), whereas differentiation among populations within each region was low ($F_{SC}$=0.04, P<0.0001;**Table 7.1**, Table 7.3). For pairwise population comparisons of the $F_{ST}$ and Jost's $D_{EST}$ see Table 7.3).

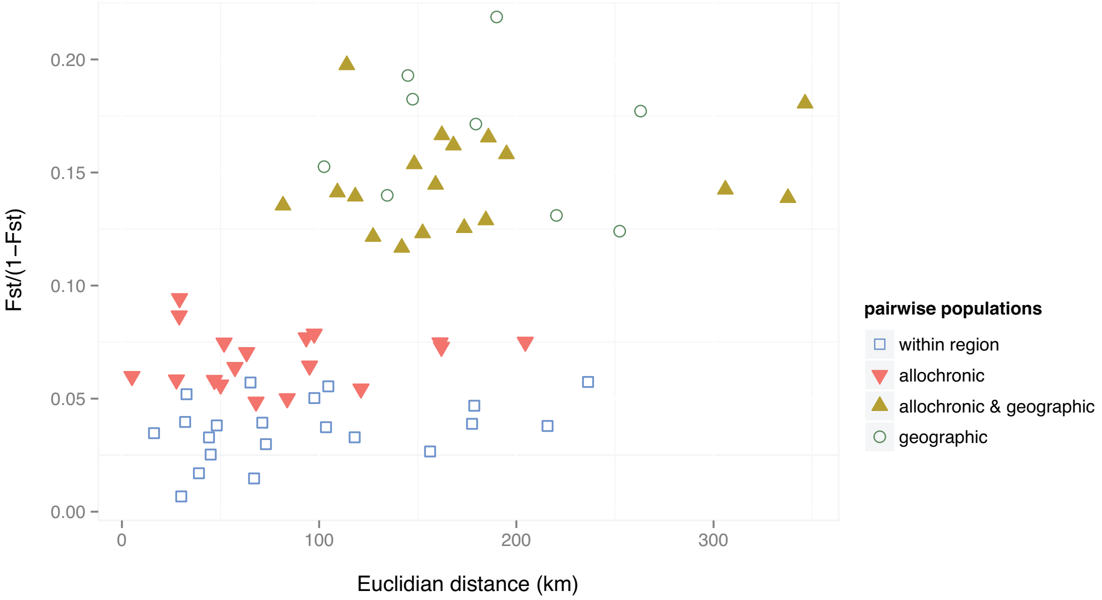

**Fig. 7.2. Isolation by distance relationship showing pairwise genetic distance ($F_{ST}/(1-F_{ST})$ between 12 populations based on 18 microsatellite loci in relation to geographic distance.** Each symbol represents pairwise population relationships for populations that are i) within each of the three regions (blue rectangle), ii) allochronically separated (red triangle), iii) allochronically and geographically separated (green triangle), and iv) geographically separated (green circle; N=312).

**Table 7.1. Results from hierarchical AMOVA testing of variation within individuals, among individuals within populations, and among populations within the three regions i) GRV early, ii) GRV late, iii) EAM late, and among the regions using (A) 18 microsatellite loci (N=312) and (B) mitochondrial cytochrome b (N=134).**

| | Sum of squares | Variance | Percentage variation | Fixation index |
|---|---|---|---|---|
| **(A) Microsatellite loci** | | | | |
| Among regions | 7625.04 | 19.79 | 12.06 | 0.11 ($F_{CT}$) |
| Among populations within regions | 3872.19 | 5.59 | 3.41 | 0.04 ($F_{SC}$) |
| Within populations | 43449.59 | 11.17 | 6.81 | 0.12 ($F_{ST}$) |
| Within individuals | 38501.00 | 128.99 | 78.64 | 0.21 ($F_{IT}$) |
| Total | 93447.81 | 164.03 | | |
| **(B) Cytochrome b** | | | | |
| Among regions | 10.399 | 0.09 | 18.27 | 0.18 ($F_{CT}$) |
| Among populations within regions | 11.139 | 0.08 | 16.68 | 0.20 ($F_{SC}$) |
| Within populations | 39.47 | 0.32 | 65.05 | 0.35 ($F_{ST}$) |
| Total | 61.01 | 0.50 | | |

**Table 7.2. (A) Expected (HE) and observed (HO) heterozygosities for 18 microsatellite loci (N=312), and (B) diversity estimates obtained from mitochondrial cytochrome b (N=134) for 12 populations of Stonechats, grouped by allochrony and geography.**

**(A)**

| **Population** | **N** | **$H_O$ (±SD)** | **$H_E$ (±SD)** |
|---|---|---|---|
| **GRV early** | | | |
| Kakamega | 19 | 0.61 ± 0.27 | 0.67 ± 0.23 |
| Mount Kenya | 30 | 0.64 ± 0.23 | 0.70 ± 0.20 |
| Kinangop | 27 | 0.72 ± 0.20 | 0.67 ± 0.24 |
| Mataara | 27 | 0.65 ± 0.22 | 0.70 ± 0.23 |
| Ngong Hills | 24 | 0.65 ± 0.23 | 0.69 ± 0.20 |
| Ol Kalou | 38 | 0.67 ± 0.24 | 0.70 ± 0.23 |
| **GRV late** | | | |
| Monduli | 25 | 0.63 ± 0.22 | 0.68 ± 0.21 |
| Mount Meru | 30 | 0.66 ± 0.19 | 0.70 ± 0.18 |
| Ngorongoro | 19 | 0.69 ± 0.23 | 0.72 ± 0.18 |
| **EAM late** | | | |
| Pare M. | 19 | 0.58 ± 0.24 | 0.58 ± 0.26 |
| Taita Hills | 28 | 0.53 ± 0.26 | 0.54 ± 0.26 |
| Usambara M. | 26 | 0.53 ± 0.25 | 0.57 ± 0.24 |

**(B)**

| **Population** | **N** | **Haplotypes** | **Haplotype diversity (±SD)** | **Nucleotide diversity (±SD)** | **Theta (θ)** |
|---|---|---|---|---|---|
| **GRV early** | | | | | |
| Kakamega | 9 | 6 | 0.833 ± 0.127 | 0.00126 ± 0.00034 | 0.00208 |
| Mount Kenya | 10 | 5 | 0.800 ± 0.100 | 0.00120 ± 0.00031 | 0.00167 |
| Kinangop | 11 | 5 | 0.618 ± 0.164 | 0.00130 ± 0.00044 | 0.00193 |
| Mataara | 11 | 5 | 0.764 ± 0.107 | 0.00185 ± 0.00035 | 0.00161 |
| Ngong Hills | 10 | 2 | 0.200 ± 0.154 | 0.00019 ± 0.00015 | 0.00033 |
| Ol Kalou | 11 | 8 | 0.927 ± 0.066 | 0.00268 ± 0.00039 | 0.00322 |
| **GRV late** | | | | | |
| Monduli | 11 | 5 | 0.855 ± 0.066 | 0.00158 ± 0.00022 | 0.00129 |
| Mount Meru | 11 | 4 | 0.782 ± 0.075 | 0.00130 ± 0.00013 | 0.00097 |
| Ngorongoro | 16 | 5 | 0.533 ± 0.142 | 0.00069 ± 0.00024 | 0.00142 |
| **EAM late** | | | | | |
| Pare M. | 11 | 2 | 0.182 ± 0.144 | 0.00069 ± 0.00054 | 0.00129 |
| Taita Hills | 12 | 4 | 0.727 ± 0.109 | 0.00086 ± 0.00019 | 0.00094 |
| Usambara M. | 11 | 4 | 0.600 ± 0.154 | 0.00117 ± 0.00034 | 0.00129 |

**Table 7.3 Pairwise $F_{ST}$ values below diagonal, Jost's $D_{EST}$ above diagonal between each population with three genetic clusters indicated by breeding onset and geographical regions.** All pairwise values are significant ($P < 0.0006$, based on 1000 iterations).

| | | **Kakamega** | **Kinangop** | **Mount Kenya** | **Mataara** | **Ngong Hills** | **Ol Kalou** | **Mount Meru** | **Monduli** | **Ngorongoro C.** | **Taita Hills** | **Pare M.** | **Usambara M.** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **GRV early** | **Kakamega** | | 0.081 | 0.046 | 0.044 | 0.099 | 0.033 | 0.072 | 0.051 | 0.043 | 0.144 | 0.198 | 0.169 |
| **GRV early** | **Kinangop** | 0.05 | | 0.055 | 0.088 | 0.076 | 0.081 | 0.075 | 0.127 | 0.110 | 0.160 | 0.198 | 0.179 |
| **GRV early** | **Mount Kenya** | 0.04 | 0.04 | | 0.017 | 0.036 | 0.005 | 0.079 | 0.073 | 0.048 | 0.127 | 0.161 | 0.148 |
| **GRV early** | **Mataara** | 0.04 | 0.05 | 0.02 | | 0.042 | 0.026 | 0.098 | 0.089 | 0.071 | 0.172 | 0.191 | 0.214 |
| **GRV early** | **Ngong Hills** | 0.07 | 0.05 | 0.04 | 0.04 | | 0.067 | 0.094 | 0.125 | 0.103 | 0.122 | 0.139 | 0.169 |
| **GRV early** | **Ol Kalou** | 0.03 | 0.05 | 0.01 | 0.01 | 0.05 | | 0.057 | 0.092 | 0.048 | 0.128 | 0.163 | 0.165 |
| **GRV late** | **Mount Meru** | 0.06 | 0.06 | 0.05 | 0.07 | 0.08 | 0.05 | | 0.031 | 0.027 | 0.139 | 0.171 | 0.160 |
| **GRV late** | **Monduli** | 0.06 | 0.07 | 0.05 | 0.06 | 0.08 | 0.05 | 0.02 | | 0.021 | 0.197 | 0.242 | 0.225 |
| **GRV late** | **Ngorongoro** | 0.05 | 0.07 | 0.05 | 0.05 | 0.07 | 0.05 | 0.03 | 0.03 | | 0.156 | 0.205 | 0.164 |
| **EAM late** | **Taita Hills** | 0.13 | 0.12 | 0.11 | 0.13 | 0.11 | 0.11 | 0.13 | 0.15 | 0.12 | | 0.045 | 0.016 |
| **EAM late** | **Pare M.** | 0.15 | 0.17 | 0.14 | 0.14 | 0.14 | 0.14 | 0.16 | 0.18 | 0.15 | 0.08 | | 0.050 |
| **EAM late** | **Usambara M.** | 0.13 | 0.13 | 0.11 | 0.14 | 0.13 | 0.12 | 0.12 | 0.15 | 0.11 | 0.04 | 0.09 | |

## Spatial connectivity between populations

To estimate the effect of habitat connectivity on the genetic structure in stonechats, we modeled the connectivity between populations based on habitat use implementing generalized functional responses (**Fig. 7.3**, (Matthiopoulos et al. 2011)) and circuit theory (**Fig. 7.4**, (McRae et al. 2008)). The spatial connectivity, expressed as resistance distance, was generally higher within than between the three regions based on the habitat suitability model (two-sided t-test, $p < 0.0001$, **Fig. 7.3**). However, the spatial connectivity between populations within the Great Rift Valley was unrelated to an early or late breeding onset (t-test, Bonferroni-corrected p=1 for both cases; **Fig. 7.3**, **Fig. 7.4**). In contrast, the populations of the Eastern Arc Mountains were spatially isolated from both the synchronic and allochronic GRV populations (Bonferroni-corrected t-tests, $p < 0.03$; **Fig. 7.3**, **Fig. 7.4**).

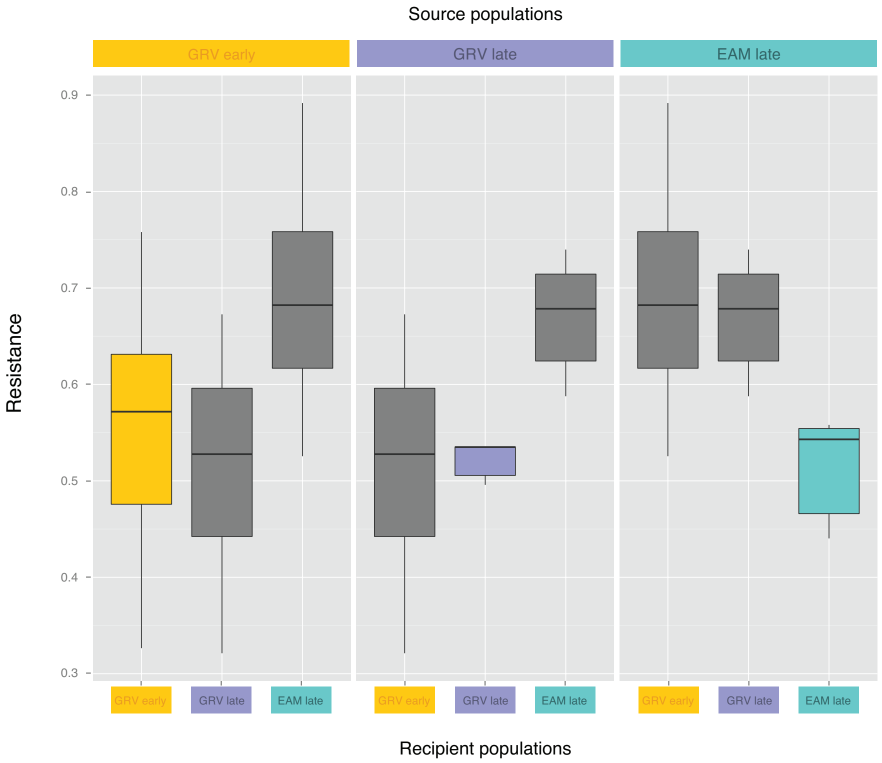

**Fig. 7.3. Resistance distance for the three regions of GRV early (yellow), GRV late (blue), and EAM late (green) based on environmental variables** (see **Table 7.8**). Shown is the resistance between the source populations of each region and all possible connections to recipient populations of each region. Colored boxplots indicate the resistance within regions. Box plots represent, from bottom to top: minimum, lower quartile, median, upper quartile and maximum.

We used the pairwise resistance distance data in a landscape genetic approach. We fitted a generalized least squares model with maximum-likelihood populations-effects (MLPE) with pairwise population matrices of the Euclidean distance, and both temporal and spatial connectivity to test inferences for the population structure of Stonechats. For the entire data set with all three regions, the best-supported model for $D_{EST}$ contained temporal and spatial connectivity (AICc=67.32, ΔAICc=0.0, ωi=0.45; Table 7.3, Table 7.4). For $F_{ST}$, similarly, the first two models included the spatial and temporal predictor separately (spatial: AICc=37.74, ΔAICc=0.0, ωi=0.27; temporal: AICc=37.89, ΔAICc=0.15, ωi=0.25; Table 7.3, Table 7.4), although in contrast to $D_{EST}$ all predictors were non-significant. Within the GRV, differences between the allochronic regions (GRV early and GRV late) were best explained in models containing only the temporal connectivity for both $D_{EST}$ and $F_{ST}$ ($D_{EST}$: AICc=86.11, ΔAICc=0.0, ωi=0.53; $F_{ST}$: AICc=80.49, ΔAICc=0.0, ωi=0.60; Table 7.3, Table 7.4). In the best-supported three models, only the temporal connectivity was significant, whereas the Euclidean distance and spatial connectivity had no significant influence (Table 7.3, Table 7.4). In contrast, when synchronic regions from the two mountain ranges were compared (GRV late and EAM late), the best supported models contained Euclidean distance for both $D_{EST}$ and $F_{ST}$ ($D_{EST}$: AICc=32.03, ΔAICc=0.0, ωi=0.65; $F_{ST}$: AICc=40.52, ΔAICc=0.0, ωi=0.73; Table 7.3, Table 7.4). Across models, relative support for top candidate models differed occasionally between $D_{EST}$ and $F_{ST}$, but the signs of regression coefficients were identical, and incongruence was mostly restricted to models with non-significant predictors.

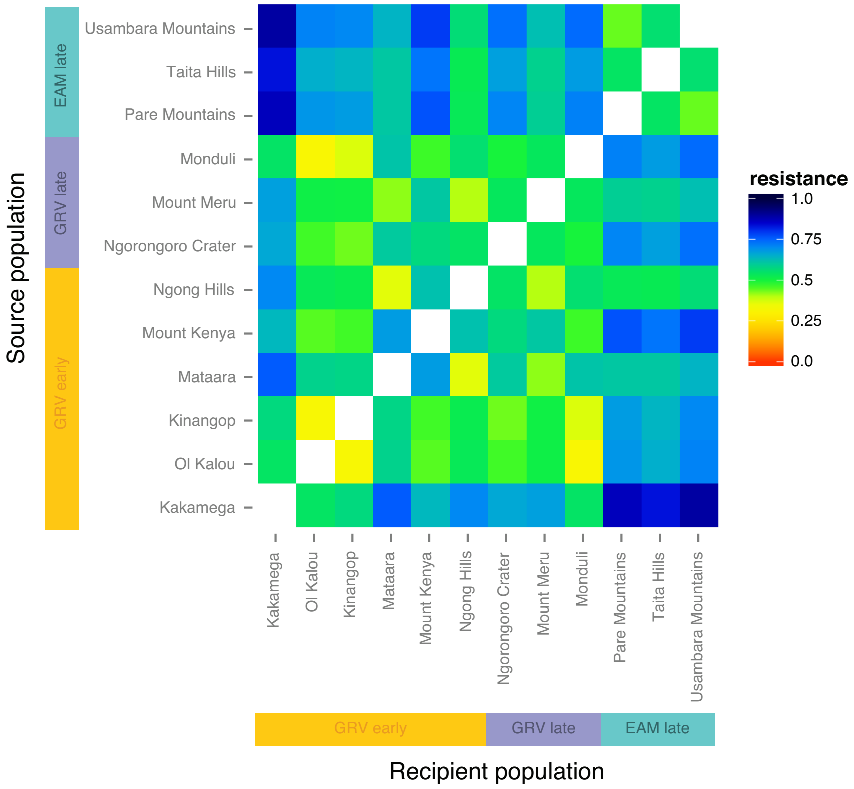

**Fig. 7.4. Resistance matrix showing all pairwise connections between 12 populations based on environmental variables** (see **Table 7.8**). The connectivity between two populations is plotted in two directions (source to recipient population and vice versa). Population origin is color coded as in **Fig. 7.1**.

**Table 7.4. Generalized least squares models with maximum-likelihood population-effects (MLPE) results showing the relationship between pairwise genetic distance ($D_{EST}$ and $F_{ST}$) and Euclidean distance, and temporal and spatial connectivity.** Shown are the comparisons between A) populations of the two geographically separated regions of the two mountain ranges, GRV late – EAM late, B) allochronic populations of the Great Rift Valley, GRV early – GRV late, and C) all 12 populations of the three regions, GRV early – GRV late – EAM late. Model selection is based on AICc with a correction for finite sample sizes.

**A)**

| | | DEST | | | | | | | | FST | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Mod** | **Variables** | **β** | **P** | **ρ** | **AICc** | **ΔAICc** | **Akaike Weight (ωi)** | **Cum. Weight** | **Log Likelihood** | **β** | **P** | **ρ** | **AICc** | **ΔAICc** | **Akaike Weight (ωi)** | **Cum. Weight** | **Log Likelihood** |
| 1 | Euclidean | 1.0191 | 0.0000 | 0.4174 | 32.03 | 0.00 | 0.65 | 0.65 | -10.01 | 0.8920 | 0.0000 | 0.3646 | 40.52 | 0.00 | 0.73 | 0.73 | -14.26 |
| 2 | Euclidean | 0.7586 | 0.0001 | 0.0000 | 34.86 | 2.84 | 0.16 | 0.80 | -6.18 | 0.6462 | 0.0052 | 0.0000 | 46.08 | 5.56 | 0.05 | 0.97 | -11.79 |
| | Spatial | 0.3227 | 0.0309 | | | | | | | 0.5142 | 0.0164 | | | | | | |
| | Temporal | 0.5056 | 0.0019 | | | | | | | 0.3433 | 0.0975 | | | | | | |
| 3 | Euclidean | 0.6505 | 0.0007 | 0.0000 | 35.67 | 3.64 | 0.10 | 0.91 | -9.50 | 0.6043 | 0.0073 | 0.1101 | 44.14 | 3.62 | 0.12 | 0.85 | -13.73 |
| | Spatial | 0.4220 | 0.0125 | | | | | | | 0.3814 | 0.0886 | | | | | | |
| 4 | Euclidean | 1.0404 | 0.0000 | 0.4143 | 35.97 | 3.94 | 0.09 | 1.00 | -9.65 | 0.9007 | 0.0001 | 0.3568 | 45.11 | 4.59 | 0.07 | 0.92 | -14.22 |
| | Temporal | 0.0934 | 0.4552 | | | | | | | 0.0467 | 0.7983 | | | | | | |
| 5 | Spatial | 0.6307 | 0.0117 | 0.0000 | 45.93 | 13.90 | 0.00 | 1.00 | -16.96 | 0.5956 | 0.0191 | 0.0000 | 46.96 | 6.44 | 0.03 | 1.00 | -17.48 |
| 6 | Spatial | 0.6524 | 0.0187 | 0.0000 | 50.52 | 18.49 | 0.00 | 1.00 | -16.93 | 0.6392 | 0.0237 | 0.0000 | 51.34 | 10.82 | 0.00 | 1.00 | -17.34 |
| | Temporal | 0.0592 | 0.8809 | | | | | | | 0.1188 | 0.6391 | | | | | | |
| 7 | Temporal | -0.1802 | 0.5204 | 0.0000 | 53.04 | 21.01 | 0.00 | 1.00 | -20.52 | -0.1158 | 0.6811 | 0.0000 | 53.33 | 12.81 | 0.00 | 1.00 | -20.67 |

**B)**

| **Mod** | **Variables** | **β** | **P** | **ρ** | **AICc** | **ΔAICc** | **Akaike Weight (ωi)** | **Cum. Weight** | **Log Likelihood** | **β** | **P** | **ρ** | **AICc** | **ΔAICc** | **Akaike Weight (ωi)** | **Cum. Weight** | **Log Likelihood** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Temporal | 0.6561 | 0.0000 | 0.2930 | 86.11 | 0.00 | 0.53 | 0.53 | -38.41 | 0.7265 | 0.0000 | 0.2937 | 80.49 | 0.00 | 0.60 | 0.60 | -35.60 |
| 2 | Temporal | 0.8428 | 0.0016 | 0.2793 | 88.03 | 1.92 | 0.20 | 0.73 | -38.01 | 0.8016 | 0.0014 | 0.2934 | 83.06 | 2.56 | 0.17 | 0.95 | -35.53 |
| | Euclidean | -0.2259 | 0.3899 | | | | | | | -0.0894 | 0.7161 | | | | | | |
| 3 | Temporal | 0.6558 | 0.0000 | 0.3039 | 88.25 | 2.14 | 0.18 | 0.92 | -38.12 | 0.7284 | 0.0000 | 0.2950 | 82.90 | 2.41 | 0.18 | 0.79 | -35.45 |
| | Spatial | 0.0873 | 0.4655 | | | | | | | -0.0583 | 0.6008 | | | | | | |
| 4 | Temporal | 0.8948 | 0.0012 | 0.2870 | 89.92 | 3.81 | 0.08 | 1.00 | -37.51 | 0.7791 | 0.0025 | 0.2951 | 85.73 | 5.24 | 0.04 | 1.00 | -35.42 |
| | Euclidean | -0.2894 | 0.2915 | | | | | | | -0.0605 | 0.8140 | | | | | | |
| | Spatial | 0.1181 | 0.3456 | | | | | | | -0.0512 | 0.6607 | | | | | | |
| 5 | Euclidean | 0.5903 | 0.0004 | 0.3150 | 96.19 | 10.08 | 0.00 | 1.00 | -43.45 | 0.6726 | 0.0000 | 0.2975 | 91.68 | 11.19 | 0.00 | 1.00 | -41.20 |
| 6 | Euclidean | 0.5883 | 0.0001 | 0.3193 | 98.84 | 12.73 | 0.00 | 1.00 | -43.42 | 0.6880 | 0.0000 | 0.2894 | 93.23 | 12.74 | 0.00 | 1.00 | -40.62 |
| | Spatial | 0.0331 | 0.8106 | | | | | | | -0.1346 | 0.3044 | | | | | | |
| 7 | Spatial | 0.0373 | 0.8277 | 0.1216 | 108.67 | 22.57 | 0.00 | 1.00 | -49.69 | -0.0632 | 0.7150 | 0.0836 | 109.31 | 28.81 | 0.00 | 1.00 | -50.01 |

C)

| | | DEST | | | | | | | | FST | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Mod** | **Variables** | **β** | **P** | **ρ** | **AICc** | **ΔAICc** | **Akaike Weight (ωi)** | **Cum. Weight** | **Log Likelihood** | **β** | **P** | **ρ** | **AICc** | **ΔAICc** | **Akaike Weight (ωi)** | **Cum. Weight** | **Log Likelihood** |
| 1 | Temporal | 1.4423 | 0.0277 | 0.2486 | 67.32 | 0.00 | 0.45 | 0.45 | -24.40 | 0.4275 | 0.3921 | 0.3501 | 39.52 | 1.78 | 0.11 | 0.82 | -10.50 |
| | Spatial | 0.1117 | 0.0394 | | | | | | | 0.0391 | 0.3513 | | | | | | |
| 2 | Temporal | 1.2430 | 0.0603 | 0.2503 | 69.43 | 2.11 | 0.16 | 0.61 | -26.75 | 0.3584 | 0.4663 | 0.3533 | 37.89 | 0.15 | 0.25 | 0.52 | -10.98 |
| 3 | Temporal | 1.4370 | 0.0290 | 0.2573 | 69.81 | 2.49 | 0.13 | 0.74 | -24.30 | 0.4320 | 0.3917 | 0.3484 | 42.13 | 4.40 | 0.03 | 1.00 | -10.46 |
| | Spatial | 0.1035 | 0.0713 | | | | | | | 0.0425 | 0.3362 | | | | | | |
| | Euclidean | 0.0563 | 0.6645 | | | | | | | -0.0278 | 0.7946 | | | | | | |
| 4 | Spatial | 0.0942 | 0.0876 | 0.2434 | 70.09 | 2.77 | 0.11 | 0.85 | -27.08 | 0.0338 | 0.4134 | 0.3482 | 37.74 | 0.00 | 0.27 | 0.27 | -10.90 |
| 5 | Temporal | 1.2643 | 0.0550 | 0.2649 | 70.78 | 3.46 | 0.08 | 0.93 | -26.13 | 0.3585 | 0.4699 | 0.3533 | 40.48 | 2.74 | 0.07 | 0.97 | -10.98 |
| | Euclidean | 0.1361 | 0.2808 | | | | | | | 0.0025 | 0.9806 | | | | | | |
| 6 | Euclidean | 0.1269 | 0.3239 | 0.2622 | 72.26 | 4.95 | 0.04 | 0.97 | -28.17 | 0.0016 | 0.9900 | 0.3514 | 38.46 | 0.73 | 0.19 | 0.71 | -11.27 |
| 7 | Spatial | 0.0856 | 0.1430 | 0.2534 | 72.50 | 5.18 | 0.03 | 1.00 | -26.98 | 0.0369 | 0.3973 | 0.3465 | 40.27 | 2.54 | 0.08 | 0.90 | -10.87 |
| | Euclidean | 0.0583 | 0.6633 | | | | | | | -0.2500 | 0.8144 | | | | | | |

## Concordance in genotypic and phenotypic traits

Populations of Stonechats differed from each other in their genetic, song and morphological traits (**Fig. 7.5**). A discriminant analysis of principal components for the 18 microsatellite loci was concordant with findings from AMOVA, revealing high levels of genetic structure between but not within the three regions (**Fig. 7.5**A). Each membership was assigned with high probability to the respective population within each region ranging from 0.73 – 1.00 ( **Table 7.5**A, **Fig. 7.5**). For the song traits, the first three principal components explained 87% (PC1=36.91, PC2=31.71, PC3=18.45; **Table 7.5**B, **Fig. 7.5**B), and for morphology traits 90% of the variation among the three regions (PC1=36.91, PC2=31.71, PC3=18.45; **Table 7.5**B, **Fig. 7.5**C). The general linear mixed models of the first principal component showed that, concordant with the genetic structure, song traits of all three regions differed from each other ( **Table 7.5**C; **Fig. 7.5**B), whereas morphology traits of allochronic populations from the Great Rift Valley were similar and only differed from the spatially isolated populations of the Eastern Arc Mountains ( **Table 7.5**C; **Fig. 7.5**C). In addition, a canonical variate analysis of the breast patch shape in males revealed similar differentiation between regions as shown for the biometric traits (**Fig. 7.6**). The breast patch shape of spatially isolated populations of the two mountain ranges, but not of the allochronic regions within the GRV, differed from each other ( **Table 7.5**C; **Fig. 7.6**).

**Table 7.5. (A) Membership probability of the Discriminant analysis of principal components (DAPC) for genotypes of 12 stonechat populations based on 18 microsatellite loci**. Principal component analyses (PCA) including factor loadings, eigenvalues, variance, and a General linear mixed model (LMM) of the first principal component for (B) seven song traits (N=196), and (C) seven morphological traits (N=349). (D) LMM for the centroid size of the breast patch shape in males (N=194). Significant differences are shown in bold.

**A)**

| **Cluster** | **Great Rift Valley - early** | | | | | | **Great Rift Valley - late** | | | **Eastern Arc Mountains - late** | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Population** | KA | KIN | KE | MA | NG | OLK | ME | MON | NGO | TAI | PA | USA |
| **membership probability** | 0.89 | 0.78 | 1.00 | 0.88 | 0.92 | 0.82 | 0.73 | 0.87 | 0.95 | 1.00 | 0.95 | 0.92 |

KA Kakamega, KIN Kinangop, KE Mount Kenya, MA Mataara, NG Ngong Hills, OLK Ol Kalou, ME Mount Meru, MON Monduli, NGO Ngorongoro Crater, TAI Taita Hills, PA Pare Mountains, USA Usambara Mountains.

**B)**

| | **PC1** | **PC2** | **PC3** |
|---|---|---|---|
| **Song** | | | |
| Song duration | -0.45 | 0.39 | 0.78 |
| No. of elements | 0.34 | -0.49 | 0.76 |
| Element rate | -0.45 | 0.77 | 0.08 |
| Peak frequency | 0.75 | -0.29 | -0.13 |
| Min. frequency | -0.24 | -0.85 | 0.17 |
| Max. frequency | 0.91 | 0.26 | 0.23 |
| Bandwidth | 0.78 | 0.61 | 0.08 |
| Eigenvalue | 2.58 | 2.22 | 1.29 |
| % variance | 36.91 | 31.71 | 18.45 |
| cumulative % variance | 36.91 | 68.62 | 87.07 |

| | | | |
|---|---|---|---|
| **Morphology** | | | |
| Body mass | 0.06 | 0.85 | 0.34 |
| Tarsus length | 0.71 | 0.32 | 0.49 |
| Wing length | 0.75 | -0.48 | 0.36 |
| Tail length | 0.82 | -.0.53 | -0.01 |
| Head length | 0.73 | -0.54 | -0.26 |
| Head-Bill length | 0.64 | 0.20 | -0.70 |
| Eigenvalue | 2.70 | 1.67 | 1.04 |
| % variance | 44.96 | 27.82 | 17.38 |
| cumulative % variance | 44.96 | 72.78 | 90.16 |

**(C)**

| | Song β (95% CI) | Biometrics β (95% CI) | Breast patch β (95% CI) |
|---|---|---|---|
| **Fixed effects** | | | |
| GRV early (intercept) | **0.43 (0.13, 0.77)** | 0.30 (-0.30, 0.90) | **8.90 (7.48, 10.33)** |
| GRV late | **-1.03 (-1.58, -0.49)** | 0.11 (-0.92, 1.11) | 0.78 (-0.92, 2.51) |
| EAM early | **-0.35 (-0.97, -0.24)** | **-1.41 (-2.47, -0.43)** | **-1.25 (0.19, 0.76)** |
| | | | |
| **Random effects** | **$\sigma^2$ (s.d.)** | **$\sigma^2$ (s.d.)** | **$\sigma^2$ (s.d.)** |
| population | 0.11 (0.33) | 0.50 (0.71) | 1.37 (1.17) |
| residual | 0.86 (0.93) | 1.30 (1.14) | 1.56 (1.25) |

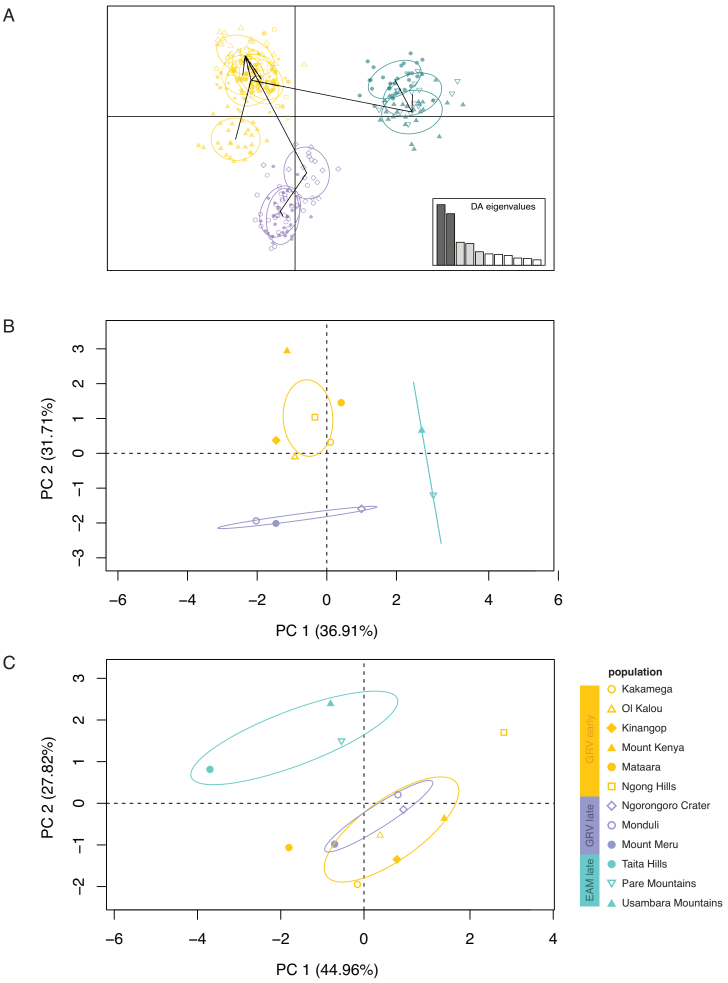

**Fig. 7.5. Identification of similarity between populations.** (A) Discriminant analysis of principal components for genotypes of 12 populations based on 18 microsatellite loci (N=312). (B) Principal component analyses for seven song traits of 11 populations (Taita Hills excluded; N=196), and (C) for seven morphological traits of 12 populations (N=349). Population origin is color coded as in **Fig. 7.1**.

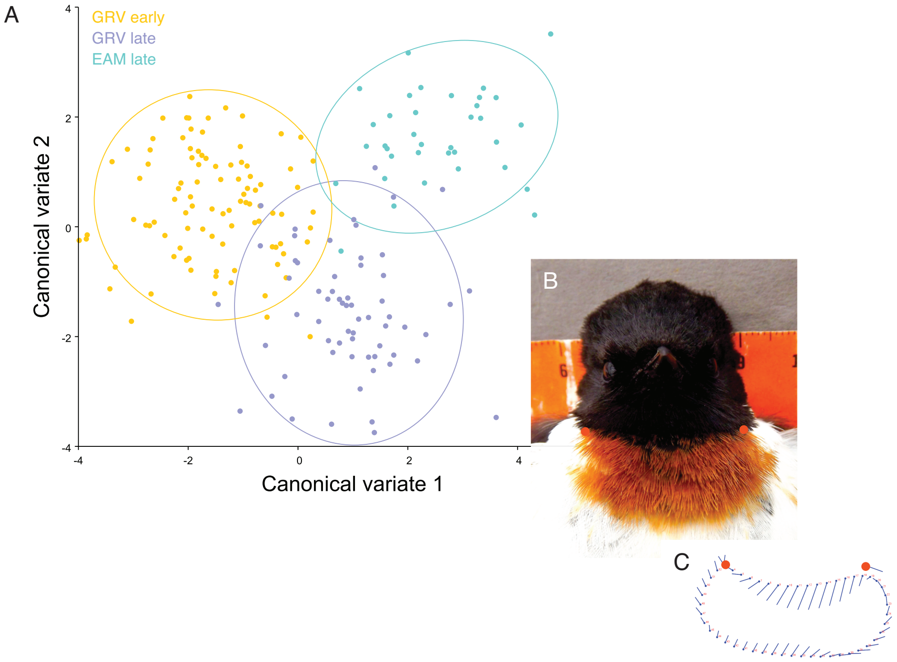

**Fig. 7.6 (A) Canonical variate analysis for the breast patch shape of three genetic clusters from i) GRV early, ii) GRV late, and iii) EAM late** (N=194), (B) location of two landmarks (red) and 50 semilandmarks (blue) describing the breast patch shape of African stonechats, and (C) lollipop diagram showing displacements at landmarks, using default settings. Population origin is color coded as in **Fig. 7.1**.

## Preference for local sexually selected traits

To investigate potential behavioral isolation between allochronically and geographically separated populations, we performed playback and decoy experiments.

### *Playback experiment*

Both sexes discriminated between origins of playbacks, thereby differing in their latency to approach (**Fig. 7.7**A) and time spent near song stimuli (**Fig. 7.7**B). The latency to approach was lowest when exposed to the local and synchronic song for females (synchronic: z=−1.92, p <0.05; **Table 7.6**A) and males (synchronic: z=−4.68, p <0.001; **Table 7.6**B). Among 40 trials for each sex, only one female and six males responded to allochronic songs at all (female: z=-3.79, p <0.001, male: z=-6.56, p <0.001, N=40; **Table 7.6**). Except for the date of playback in females, all other factors, i.e. population membership, breeding stage, trial order and time of day, had no significant effect on the latency to approach in both sexes (**Table 7.6**). Females and males also spent significantly more time within 5 m to the stimuli when presented with the local song than with the non-local synchronic (female: estimate=-2.00, CI=-2.44, -1.52; male: estimate=-1.50, CI=-1.89, -1.08), allochronic (female: estimate=-4.06, CI=-4.33, -3.75; male: estimate=-2.81, CI=-3.22, -2.35), European (female: estimate=-4.23, CI=-4.47, -3.95; male: estimate=-4.10, CI=-4.34, -3.82), and control song (female: estimate=-4.32, CI=-4.52, -4.11; male: estimate=-4.16, CI=-4.39, -3.91; **Table 7.7**A and B, **Fig. 7.7**B).

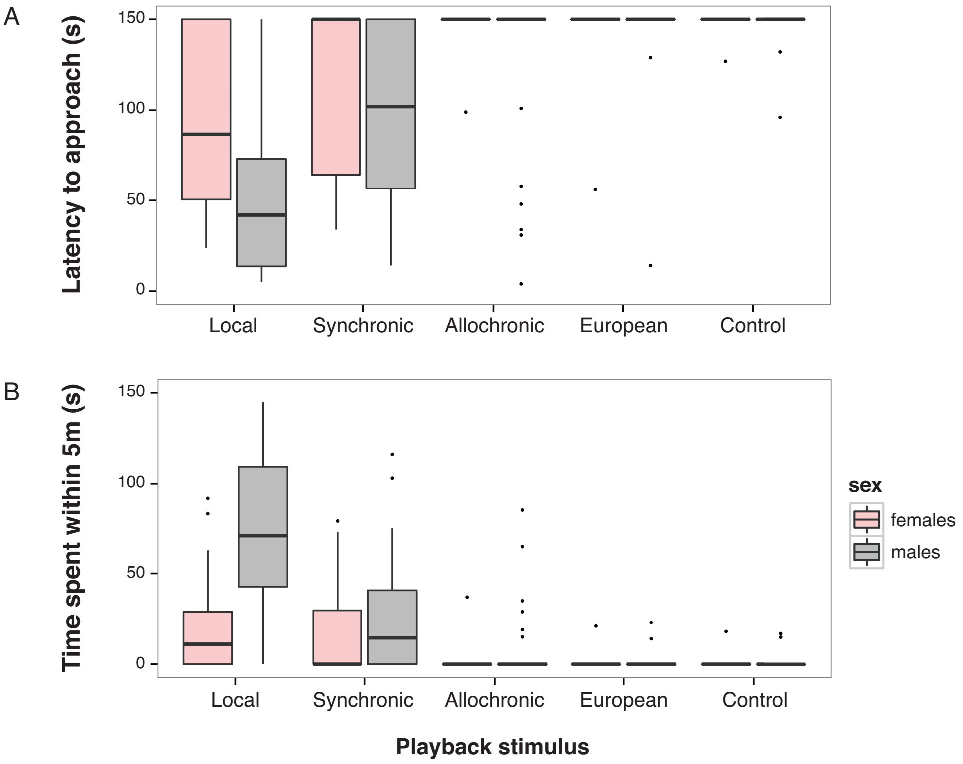

**Fig. 7.7 Behavioral response of female (N=40) and male Stonechats (N=40) during playback**. (A) latency to approach the playback, plotted by origin of the stimulus; (B) time spent within 5 m to song of African stonechats *S. torquata axillaris* from (i) local, (ii) synchronic, and (iii) allochronic populations, (iv) European stonechats *S. torquata rubicola*, and (v) control stimuli. Box plots as in **Fig. 7.3**.

**Table 7.6. Latency to approach a simulated territorial intruder to within 5 m.** Results of cox mixed-effects model of factors that influence latency, with estimates, hazard ratio, standard error, z-value, and p-value fitted by maximum likelihood. Results for responses to playback of (A) females and (B) males, and to decoys in (C) females and (D) males. Estimates refer to differences from the intercept estimate, which represents the latency to approach stimuli of the local population (not shown). Subjects were included as random intercepts, and populations as random slopes to control for repeated measures. 'Significant' differences are shown in bold.

| | fixed effects | estimate | hazard ratio | s.e.m | z | p |
|---|---|---|---|---|---|---|
| **playback** | | | | | | |
| (A) females | synchronic | -0.61 | 0.54 | 0.32 | -1.92 | 0.055 |
| | **allochronic** | -3.88 | 0.02 | 1.02 | -3.79 | **<0.001** |
| | **European** | -3.88 | 0.02 | 1.03 | -3.79 | **<0.001** |
| | **control** | -3.84 | 0.02 | 1.02 | -3.75 | **<0.001** |
| | population | -1.13 | 0.32 | 0.61 | -1.87 | 0.061 |
| | breeding stage | -0.01 | 0.99 | 0.08 | -0.16 | 0.870 |
| | trial order | -0.12 | 0.88 | 0.11 | -1.13 | 0.260 |
| | **date** | -0.38 | 0.69 | 0.14 | -2.61 | **0.009** |
| | time | 0.02 | 1.02 | 0.01 | 1.48 | 0.140 |
| (B) males | **synchronic** | -1.37 | 0.25 | 0.29 | -4.68 | **<0.001** |
| | **allochronic** | -3.07 | 0.05 | 0.47 | -6.56 | **<0.001** |
| | **European** | -4.33 | 0.01 | 0.74 | -5.82 | **<0.001** |
| | **control** | -4.28 | 0.01 | 0.74 | -5.75 | **<0.001** |
| | population | -0.70 | 0.50 | 0.63 | -1.12 | 0.260 |
| | breeding stage | 0.04 | 1.04 | 0.10 | 0.36 | 0.720 |
| | trial order | -0.11 | 0.89 | 0.09 | -1.21 | 0.220 |
| | date | -0.08 | 0.93 | 0.15 | -0.49 | 0.620 |
| | time | 0.00 | 1.00 | 0.01 | -0.09 | 0.930 |
| **decoy** | | | | | | |
| (C) females | **allochronic** | -1.10 | 0.33 | 0.33 | -3.29 | **<0.001** |
| | **European** | -3.61 | 0.03 | 0.75 | -4.82 | **<0.001** |
| | **control** | -3.80 | 0.02 | 0.76 | -5.01 | **<0.001** |

| | fixed effects | estimate | hazard ratio | s.e.m | z | p |
|---|---|---|---|---|---|---|
| | population | -0.47 | 0.62 | 0.78 | -0.60 | 0.260 |
| | breeding stage | -0.23 | 0.79 | 0.22 | -1.07 | 0.720 |
| | **trial order** | 0.38 | 1.47 | 0.17 | 2.20 | 0.220 |
| | **date** | -0.19 | 0.83 | 0.08 | -2.33 | 0.620 |
| | time | 0.002 | 1.00 | 0.005 | -0.39 | 0.930 |
| (D) males | **allochronic** | -0.88 | 0.41 | 0.29 | -3.09 | **0.002** |
| | **European** | -2.91 | 0.05 | 0.49 | -5.89 | **<0.001** |
| | control | NA | NA | NA | NA | NA |
| | **population** | -1.38 | 0.25 | 0.66 | -2.09 | **0.036** |
| | breeding stage | -0.01 | 0.99 | 0.17 | -0.05 | 0.960 |
| | trial order | 0.03 | 1.03 | 0.14 | 0.24 | 0.810 |
| | **date** | -0.14 | 0.87 | 0.06 | -2.23 | **0.026** |
| | time | 0.00 | 1.00 | 0.00 | -0.95 | 0.340 |

### *Decoy experiment*

In response to the decoy, females and males discriminated significantly between origins of the stimulus (**Fig. 7.8**). Both sexes had a lower latency to approach local decoy stimuli compared to the allochronic (female: z=-3.29, p <0.001, male: z=-3.09, p <0.001), European (female: z=-4.82, p <0.001, male: z=-5.89, p <0.001), and control stimuli (female: z=-5.01, p <0.001, male: NA; **Table 7.6** C and D, **Fig. 7.8**A). In females, trial order and date of experiment, but not population membership, breeding stage, and time of day had a significant effect on the latency to approach the stimuli (**Table 7.6**C). In males, population membership and date of experiment, but not breeding stage, trial order, and time of day had a significant effect on the latency to approach the stimuli (**Table 7.6**D). For both sexes, the time spent within 5 m close to stimulus was longest when exposed to the local decoy and decreased for the allochronic (female: estimate=-1.50, CI=-1.96, -1.01; male: estimate=-0.58, CI=--0.99, -0.16), European (female: estimate=-3.60, CI=-3.98, -3.16; male: estimate=-2.18, CI=-2.68, -1.60), and control decoy (female: estimate=-4.47, CI=-4.59, -4.35; male: estimate=-4.60, CI=-4.60, -4.60; **Table 7.7**C and D, **Fig. 7.8**B). In addition, males attacked the local decoy significantly more often than all other stimuli (**Table 7.7**E, **Fig. 7.8**C). In contrast, females never attempted to attack the decoy.

**Table 7.7. Time spent within 5 m of a territorial intruder simulated by playback.** Results of a generalized linear mixed model with estimates and credible intervals using WinBUGS for responses to playback of (A) females and (B) males, to decoys in (C) females and (D) males, and (E) for the number of attacks in males. Stimulus is defined as a random factor to compare paths of all stimuli and thus correct for multiple testing. A behavioral response differs significantly from the local population if its credible intervals do not include the mean of the local population. Significant results are shown in bold.

| | Time spent within 5 m | | Time spent within 5 m | | Number of attacks |
|---|---|---|---|---|---|
| | Playback β (95% CI) | | Decoy β (95% CI) | | Decoy β (95% CI) |
| **fixed effects** | **(A) female** | **(B) male** | **(C) female** | **(D) male** | **(E) male** |
| local | **-2.00 (-2.33, -1.64)** | -0.23 (-0.59, 0.13) | **-0.54 (-0.98, -0.10)** | 0.19 (-0.24, 0.61) | **40.57 (27.83, 52,69)** |
| synchronic | **-2.00 (-2.44, -1.52)** | **-1.50 (-1.89, -1.08)** | NA | NA | NA |
| allochronic | **-4.06 (-4.33, -3.75)** | **-2.81 (-3.22, -2.35)** | **-1.50 (-1.96, -1.01)** | **-0.58 (-0.99, -0.16)** | **-5.84 (-8.81, -2.70)** |
| European | **-4.23 (-4.47, -3.96)** | **-4.10 (-4.34, -3.82)** | **-3.60 (-3.98, -3.16)** | **-2.18 (-2.68, -1.60)** | **-12.80 (-15.84, -9.71)** |
| control | **-4.32 (-4.52, -4.11)** | **-4.16 (-4.39, -3.91)** | **-4.47 (-4.59, -4.35)** | **-4.60 (-4.60, -4.60)** | **-13.77 (-16.76, -10.75)** |

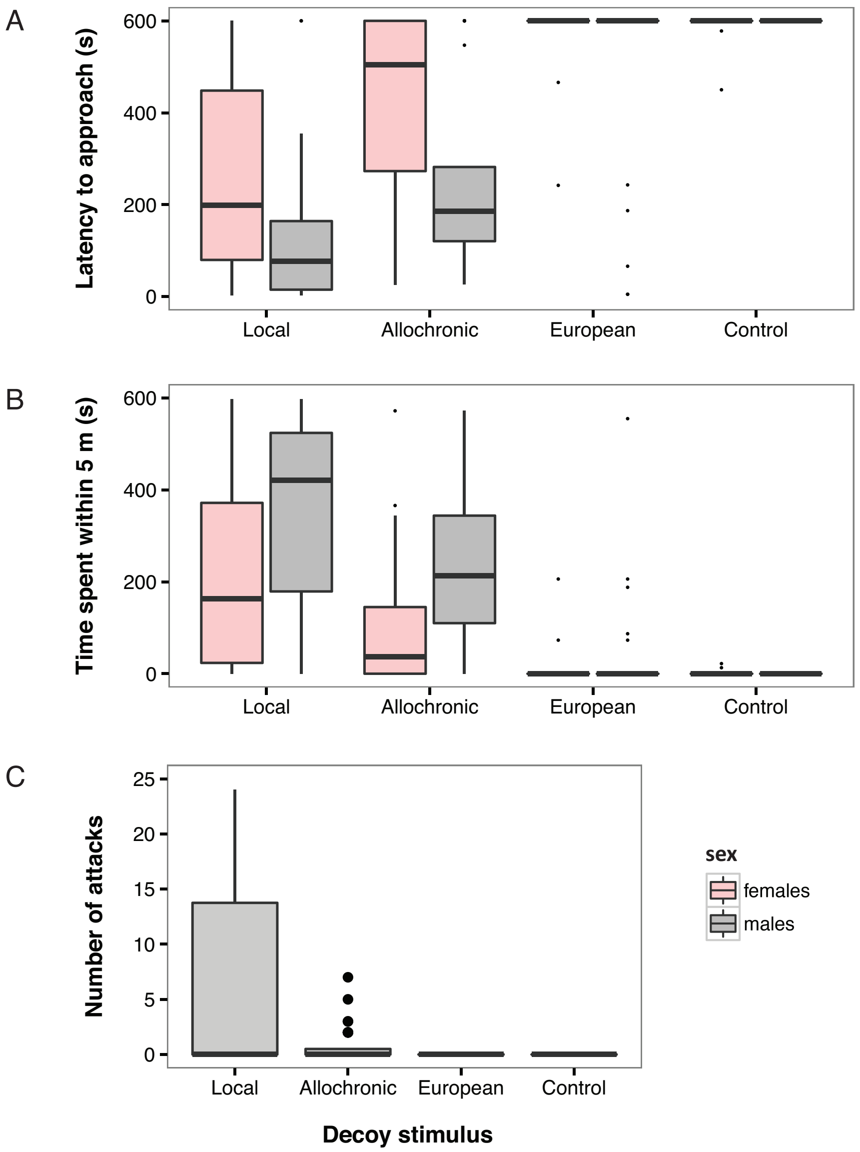

**Fig. 7.8. Behavioral response in females (N=38) and males (N=38) during decoy experiments** for (A) latency to approach, (B) time spent within 5 m, and (C) number of attacks (only in males) to i) local, ii) and allochronic populations of African stonechats, iii) European stonechats, and iv) control stimuli. Box plots as in **Fig. 7.7**.

# Discussion

We set out to integrate the relative contributions of temporal, spatial, and behavioral factors to isolation and its evolutionary implications, leading to population divergence. Populations of African stonechats showed considerable substructure and concordance in patterns of genotypic and phenotypic traits between three regions, which are separated by seasonal timing and geography, respectively. Our findings strongly suggest potential allochronic and geographic reproductive barriers restricting gene flow between regions. Allochronic reproductive isolation mechanisms thus may drive evolutionary diversification and ultimately speciation, but have been largely ignored. Traditional studies emphasize the geographic separation as a necessary prerequisite to speciation. One prominent example for geographic isolation barriers is the ring species of the Greenish warbler, *Phylloscopus trochiloides*. Reproductively isolated populations of this species coexist despite being connected by apparently gradual variation around a geographic barrier (Irwin et al. 2005). Such topographic conditions but also the ability to move through a landscape are thought to shape evolutionary processes (Garant et al. 2005). In some instances, divergence may be promoted to a substantial extent by dispersal events, depending on organism-specific abilities to persist and disperse in a landscape. For example, short-distance dispersal in a continuously distributed species is expected to cause a pattern of isolation by distance (Greenwood and Harvey 1982). In contrast, in Stonechats we found little evidence for isolation by distance. Instead, there was an influence of geographic barriers, such as the highly unsuitable habitat between the two mountain ranges in African stonechats, and of differences in reproductive timing.

A previous study on captive *Saxicola* species revealed that birds did not modify timing in response to a slightly allochronic mate, resulting in decreased reproductive success (Gwinner et al. 1995b; Helm et al. 2009; Bradshaw and Holzapfel 2010). Thus, species such as Stonechats, in which timing of breeding is highly heritable, appear to have limited phenological plasticity, implying that allochronic populations would experience highly reduced effective gene flow even if they were connected by dispersal (Hendry and Day 2005; Helm et al. 2009). We tested the environmental connectivity, i.e. the ability to disperse, between allochronic populations along the escarpment of the Great Rift Valley. Remarkably, allochronic populations were genetically differentiated although the spatial connectivity did not differ from synchronic populations. This is a strong indication that allochronic breeding can contribute more to isolation than distance or geographic factors, supporting the "Temporal Environment" ($E_T$) hypothesis. In contrast, the topographic conditions as well as isolation by distance seemed to determine the genetic diversity between the synchronically breeding but spatially separated populations of the two mountain ranges, GRV late and EAM late, thereby supporting the "Spatial Environment" ($E_s$) hypothesis. Time and space, therefore, can both separately or interactively contribute substantially to reproductive isolation.

Our study involved analyses of two classes of molecular markers, mtDNA and microsatellites. Both led to a similar signal of genetic structure among the regions, but we also found two disparities. Firstly, in addition to the three genetic clusters, the Ngorongoro crater population (GRV late) formed a fourth distinct lineage based on mtDNA. Here, the mode of inheritance needs to be considered, since genetic diversity indices from either microsatellites or mtDNA depend on a complex set of conditions (reviewed in (Karl et al. 2012)). In case of discordance between these markers, patterns can most likely be attributed to adaptive introgression of mtDNA, demographic disparities and sex-biased asymmetries (Toews and Brelsford 2012). The most likely explanation for the discrepancy between both markers is the matrilineal inheritance of mtDNA, which may indicate male-biased dispersal from the Ngorongoro crater population. Secondly, the most common haplotype was shared between early and late breeding populations of the GRV, which suggests incomplete lineage sorting, and thus a rather recent diversification between allochronic populations. In addition, preliminary results based on the mitochondrial cytochrome b indicate an estimated divergence time between allochronic populations of at least 100,000 years ago, in contrast to the considerably earlier divergence time between spatially separated populations dating to about 600,000 years ago.

The integrative approach of our study allowed us to investigate behavioral mechanisms that may be driving the divergence between stonechat populations. We found strong evidence for geographic differentiation of sexually selected traits in African stonechats that may affect the reduced gene flow between allochronic populations. Song but not morphology differed significantly in allochronic populations, whereas song as well as morphology differed between the two mountain ranges. Consistently, both sexes distinguished local morphological and especially acoustic phenotypes from those of allochronic populations. In contrast, in a study on behavioral responses to local and foreign dialects of rufous-collared sparrows, *Zonotrichia capensis*, females preferred their natal song dialect to the dialect of a geographically close population that was separated by an unsuitable higher elevation habitat (Danner et al. 2011). Males of this species showed similar territorial responses to all conspecific dialects with no consistent difference with respect to distance, making male territoriality uninformative for estimating reproductive isolation. Although the fine acoustic discrimination ability in Stonechats suggests potential behavioral barriers, its implications for geographic isolation are not fully clear, partly depending on song plasticity, and ultimately on the mechanisms involved in song learning. In passerine birds, song is typically learned during a sensitive period early in life. In species like Stonechats that show geographic discrimination, males that subsequently disperse into ranges of other populations would face reduced mating prospects if an acoustic signature of the natal population remains in their repertoire (reviewed in (Podos and Warren 2007). Our data demonstrate that variation in sexually selected traits may contribute to isolation over relatively short distances, driven by allochrony and barriers, and thereby foster local adaptation. Based on our findings, we propose that song, if seasonally expressed and seasonally acquired, can substantially reinforce allochronic isolation. Maintenance or further divergence of differences in song could lead to assortative mating, reproductive isolation, and potentially speciation.

Our findings revealed substantial contributions of temporal and spatial isolation to population divergence in African stonechats. Considerable differences in the timing of breeding between populations on a local scale may promote population divergence and ultimately incipient ecological speciation processes. In a changing world, such rigid reproductive schedules could become costly and make it hard for organisms to keep track of seasonality (Helm 2009). This might imply severe fitness consequences. Nevertheless, it also emphasizes the importance of studies on timing of breeding for conservation efforts in an era of climate change.

# Materials and Methods

## Subjects

African Stonechats, *Saxicola torquata axillaris*, are distributed at high altitudes (1389-2065 mamsl) in Kenya and Tanzania (from 00°16'13"N, 034°50'32"E to -04°30'51"S, 038°12'39"E; (Brewer 2003)). We focused on twelve sedentary populations, which differ in their spatial distribution and timing of reproduction (**Fig. 7.1**). We collected samples from two major mountain ranges, the Great Rift Valley (GRV) and the Eastern Arc Mountains (EAM; February 12th 2012 – May 05th 2013). Previous studies determined two distinct patterns of breeding time concordant with the two major rainy seasons in Eastern Africa (Dittami and Gwinner 1985). At Lake Nakuru (Kenya), Stonechats initiate breeding at the onset of the rains in March, whereas those on Mount Meru (Tanzania), about 120 km south of Lake Nakuru, start breeding in October. We confirmed that our study populations follow the same pattern, and thus are referred to as populations with an ‘early’ breeding onset (March, n=6), or a ‘late’ breeding onset (October, n=6), respectively. The Stonechats breeding season lasted approximately three months.

## Mitochondrial analysis

A region of the cytochrome b gene was amplified for 134 individuals, and sequenced using primers MT-A3 and MT-F2 (Wink et al. 2002), H4A (Harshman 1996), L14841 (Kocher et al. 1989), and H15767 (Edwards et al. 1991) following the conditions described in Illera (Illera et al. 2008). The final product was analyzed on a Perkin Elmer ABI 3700 automated sequencer. Sequences were aligned by eye using BioEdit v7.2.5 (Hall 1999). Genetic differentiation between Stonechat populations was examined using analyses of molecular variance (AMOVAs) as implemented in ARLEQUIN v3.515 (Excoffier et al. 2010). We tested significance of AMOVA analyses using 10,000 random permutations. The number of haplotypes, haplotype diversity (h, (Nei 1987)), the degree of polymorphism ($\pi$, (Nei 1987)) and theta ($\theta$, (Tajima 1996)) values for each population were calculated using the software DnaSP version 5.10.01 (Librado and Rozas 2009). Phylogenetic relationships among mitochondrial sequences were determined through haplotype network using the program TCS v1.21 (Clement et al.

2000). We used the default limit of 5% and missing data or gaps were treated as a 5th state (in addition to the four different nucleotides).

## Microsatellite analysis

DNA was extracted from blood using a standard ammonium acetate precipitation method and amplified for 18 fluorescently labeled microsatellite loci by standard PCR (Mortega et al. 2015). Fragments were analyzed on an ABI 3730 sequencer (Applied Biosystems) and sized according to Rox 500 internal standard in GeneMapper version 4.0 (Applied Biosystems, Darmstadt, Germany). We randomly duplicated 15% of all genotypes and ran all duplicates in Pedant v1.0 (Johnson and Haydon 2007, 2009). Based on the estimated heterozygosity of 0.84, we then calculated confidence intervals of the maximum likelihood of allelic dropout (0.0 – 0.02) and false allele error rate (0.0 – 0.01). We tested for Hardy-Weinberg-Equilibrium by population and by locus as well as linkage disequilibrium using Genepop on the web version 4.2 (Rousset 2008). Including relatives has the potential to influence various types of population-genetic analyses. Thus we identified pairs of close relatives calculating maximum likelihood estimates of relatedness with ML-Relate (Kalinowski et al. 2006). This revealed six pairs of close relatives. We retained the individual with more amplified microsatellite loci in each pair, and excluded the other from the analyses. We analyzed population structure with STRUCTURE version 2.3.4 (Falush et al. 2003). Replicate runs of STRUCTURE used a burn-in period of 500,000 iterations followed by 1,000,000 MCMC iterations from which estimates were obtained. All runs were based on the admixture model, in which each individual is assumed to have ancestry in multiple genetic clusters, using the F model of correlated allele frequencies across clusters. We performed ten replicate runs for each value of the number of clusters K from one to 15 using data from all populations. The number of clusters that best fit the data was identified with the Evanno method in the web-based HARVESTER software version 0.693 (Earl and vonHoldt 2012). We optimally aligned replicates to account for label switching and genuine multimodality in population genetic cluster analysis, using the full search algorithm in CLUMPP version 1.1.2 (Jakobsson and Rosenberg 2007). Individual membership coefficients of STRUCTURE results were graphically visualized using DISTRUCT (Rosenberg 2004). We used the dataset and significance assessed with 1000 permutations to compute Weir & Cockerham families of estimators of F-statistics and tested them using randomization methods in Fstat version 2.9.3.2 (Weir and Cockerham 1984). We also determined genetic diversity indices $D_{EST}$ using the software SMOGD, which are based on the effective number of alleles rather than on the expected heterozygosity as $F_{ST}$ (Crawford 2010). An AMOVA with 10 000 permutations was conducted to test if genetic structure was present within compared to among populations and regions using ARLEQUIN v3.515 (Excoffier et al. 2010). Finally, we carried out a discriminant analysis of principal components (DAPC) using the R package *adegenet* (Jombart 2012).

## Landscape genetic analysis

### *Data preparation*

We included the following environmental data for the modeling of habitat use: half-monthly NDVI data for the complete study period, as well as monthly precipitation, precipitation seasonality (coefficient of variation for monthly precipitation per year) and monthly temperature (mean, minimum, and maximum) from available climate models (**Table 7.8**). To enrich absence and presence locations with the environmental information, we used bilinear interpolation to approximate the conditions at the exact locations. One advantage of the generalized functional responses (Matthiopoulos et al. 2011), which we employed for modeling habitat use, is that differences in the conditions available to different populations can be taken into account by estimating the coefficients as functions of availability. To determine local availabilities, we used the conditions prevailing within the individual's territories, and the area directly surrounding them for each location in the dataset. We used the maximum estimates for the species described by Urquhart (Brewer 2003) as an approximation of territory size (7 ha). To capture the area directly surrounding the territories, we doubled the radius of the imaginary territories and extracted the conditions for this area (88 ha). We calculated the mean value for all locations of a respective population, and used this as the local available habitat.

### *Generalized functional responses*

To model habitat use, we used generalized functional responses as described in Matthiopoulos et al. (**Fig. 7.9**; (Matthiopoulos et al. 2011)). Being limited by 555 locations (417 presences, 138 absences) of twelve populations available, we were only able to incorporate five out of nine environmental variables into a single model (**Table 7.8**). For this reason, we first built a full model including all variables but ignoring local differences using a binomial general linear mixed model (LMM, R package *lme4* (Bates et al. 2012)). We then built generalized response function models with all potential combinations of one to five environmental variables, and calculated the AIC. We used the 5%-Quantile to identify the best models and used these for spatial predictions of habitat use.

**Table 7.8. Environmental data used to model habitat use.** For each data product, its spatial resolution and source are listed.

| Product | Description | Spatial resolution | source |
|---|---|---|---|
| Aster GDEM | Digital elevation model | 1 arc-second | USGS |
| Precipitation | Monthly rainfall | 30 arc-seconds | www.worldclim.org |
| BIO15 | Precipitation seasonality | 30 arc-seconds | www.worldclim.org |
| Temperature | Monthly mean, minimum, and maximum temperature | 30 arc-seconds | www.worldclim.org |
| MOD13A2.005 | 16-day NDVI | 30 arc-seconds | NASA |

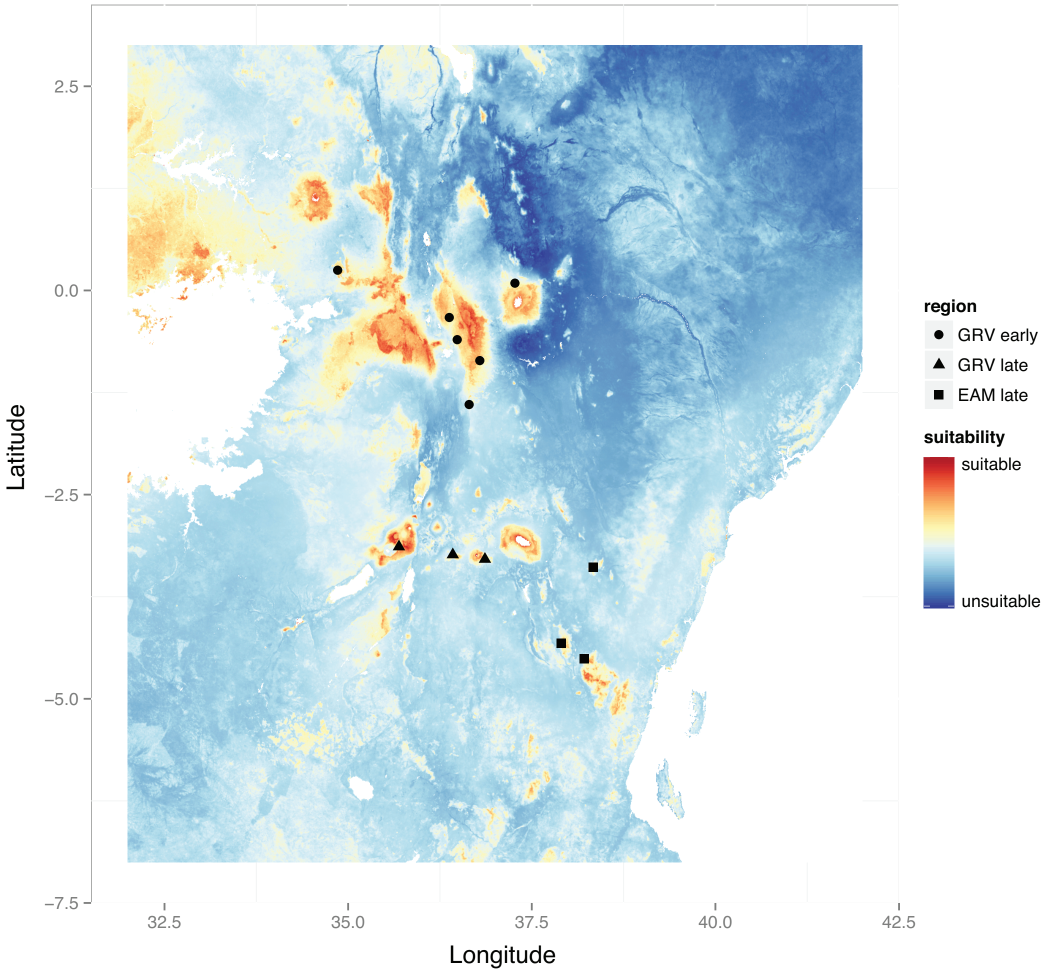

**Fig. 7.9. Predicted habitat suitability for the three regions in East Africa,** i.e. GRV early, GRV late, and EAM late calculated from generalized functional responses based on environmental variables (see **Table 7.8**). Red indicates highly suitable habitat, dark blue indicates unsuitable habitat.

### *Circuit theory*

We employed Circuitscape v4.0, which exploits electronic circuit theory to estimate connectivity between points on complex landscapes, to estimate the habitat connectivity between populations (McRae et al. 2008). Connectivity is defined as the resistance distance (Klein and Randić 1993; McRae et al. 2008), which incorporates both the minimum resistance and the availability of alternative routes. The use of circuit theory thus has an advantage over e.g. least-cost paths, as all potential paths contribute to the resulting connectivity, rather than just the optimal route. With standard settings, we calculated pairwise resistances for all populations using the inverted final habitat usage map as a resistance landscape.

*Maximum-likelihood population-effects model*

For the landscape genetic modeling, we used three pairwise population matrices, i.e. the Euclidean distance, defined as the geographic distance between populations, the temporal connectivity, defined as the minimal number of days between respective breeding peaks (average date of first egglaying), and the spatial connectivity, defined as the environmental resistance distance calculated with Circuitscape. To assess the correlations of Euclidian distance, and temporal and spatial connectivity variables, with genetic distance, we applied linear models fit by generalized least squares with maximum-likelihood population-effects (MPLE) that explicitly accounts for non-independence of values in regressions on distance matrices (gls, R package *nlme* (Pinheiro et al. 2008), (Clarke et al. 2002; Van Strien et al. 2012)). Parameter estimation was performed using restricted maximum likelihood (REML). All predictor matrices were centered around their mean prior to analysis. We created MLPE models using either $D_{EST}$ or $F_{ST}$ as the response variable and all combinations of Euclidian distance, temporal and spatial connectivity variables as fixed effects. The top models for each genetic distance measure was selected using goodness-of-fit as determined by both the Akaike information criterion corrected for finite sample size (AICc) and the Bayesian information criterion (BIC) (AICc, R package *AICcmodavg* (Mazerolle 2014)).

## Acoustic analysis

We recorded ten minutes of dawn song from eleven populations (time: 0350 – 0600, N=194; Table 7.10; for Taita Hills, song recording was not possible due to time constraints). We used a Marantz PMD 661 solid state recorder (Osnabrück, Germany) and Sennheiser ME66/K6 directional microphones (Georgsmarienhütte, Germany), and analyzed songs (sampling frequency: 44.1 kHz; resolution: 16 bit) using the software Avisoft Sound Analysis Pro, v5.1.09 (Raimund Specht, Berlin, Germany), as described in (Mortega et al. 2014). Briefly, we determined song duration, number of elements per song, element rate (number of elements per second), minimum and maximum frequency, peak frequency (frequency of the highest amplitude sound), and bandwidth. With the automatic parameter measurements setup, we obtained the minimum and maximum frequencies at a standard decibel threshold (here −20 dB, total option) below the peak in the power spectra (Zollinger et al. 2012). We used principal component analyses (PCA, R package *FactoMineR* (Husson et al. 2014)) to compare song traits between populations. We then tested the first principal component in a general linear model (LM, R package *lme4* (Bates et al. 2012)) to identify the change in song traits over geographic distance of song origins from the local population.

## Morphological analysis

We calipered the following biometric measurements of birds from all populations (1) tail, (2) wing, (3) tarsus, (4) bill, (5) head + bill length, and (6) body mass (N=349; (Eck et al. 2011)) and analyzed the data as explained above for song traits.

We digitized two landmarks and 50 semilandmarks from standardized photographs of the breast patch in TPSDIG v2.12 by a single investigator (KGM, (Rohlf 2010), **Fig. 7.6**) to describe the shape. We analyzed these landmarks with classifier variables ($N_{region}$=GRV early 96, GRV late 36, EAM late 62) in MORPHOJ v1.00j using least-squares Procrustes superimposition, where the configuration of landmarks for each specimen was scaled to unit centroid size, translated to a common position and rotated to minimize Procrustes distances between all landmark configurations (Klingenberg 2011). To assess the total amount of variation in breast patch shape between regions, we used canonical variates analysis CVA for visualization and analyzed individual centroid sizes with a general linear mixed model fitted by maximum likelihood methods (LMMs, R package *lme4* (Bates et al. 2012); (Klingenberg 2003)).

## Playback and decoy experiments

Stimulus preparation and experimental design is detailed by Mortega (Mortega et al. 2014). In both regions of the Great Rift Valley, we conducted playback (GRV early: March 19th – April 2nd 2012; GRV late: Nov 28th – Dec 2nd 2012; N=40 for each sex) and decoy experiments (GRV early: April 17th – April 24nd 2012; GRV late: Nov 17th – Nov 28nd 2012; N=38 for each sex) by simulating a territorial intrusion using phenotypes of different geographic origin. Each experiment was conducted by first catching and withholding one mate of a pair, and meanwhile testing the other. For the playback, stimulus strings consisted of songs from the local, synchronic and allochronic populations of similar geographic distance (about 200 mi) to the focal population, as well as songs of European stonechats (*S. torquata rubicola*, Düffel, Germany; Fig. 7.10B). Following the rationale of Grant and Grant (Grant and Grant 2002), we used song of heterospecific Winter wrens, *Troglodytes hiemalis*, as a control (www.macaulaylibrary.org ; recorded in NY State, United States; Fig. 7.10C) We generated a unique stimulus for each randomized trial (Kroodsma 1989). Decoy experiments were conducted without synchronic stimuli because taxidermic mounts were only available from one population for each region. We used Great tits, *Parus major*, as a control. We observed behavioral responses from a distance of 30-40 m and documented them continuously by dictating to the Marantz PMD 661. We quantified behavior using standard descriptors of responses to territorial intrusions and mate attraction including studies on Stonechats (Canoine and Gwinner 2002). Specifically, we measured i) the latency of a bird to approach the playback or decoy to within 5 m, ii) the time a bird spent within this 5 m zone, and iii) the number of male attacks of the decoy (Brewer 2003). Because data on the time spent within 5 m were zero-inflated, we analyzed them by generalized linear mixed model with a beta distribution and stimulus as random factor using WinBUGS 1.4 (GLMM, R package R2WinBUGS). We defined stimulus as a random factor to compare paths of all stimuli, and thus correct for multiple testing. The response number of attacks was analyzed with a general linear mixed model fitted by maximum likelihood methods (LMMs, R package lme4 (Bates et al. 2012)). Populations were included as random intercepts to control for repeated measures. Predictions from the general and generalized linear mixed models (Bayesian methods) were calculated as the median of their posterior distributions, and the 2.5 and 97.5% credible intervals (CI).

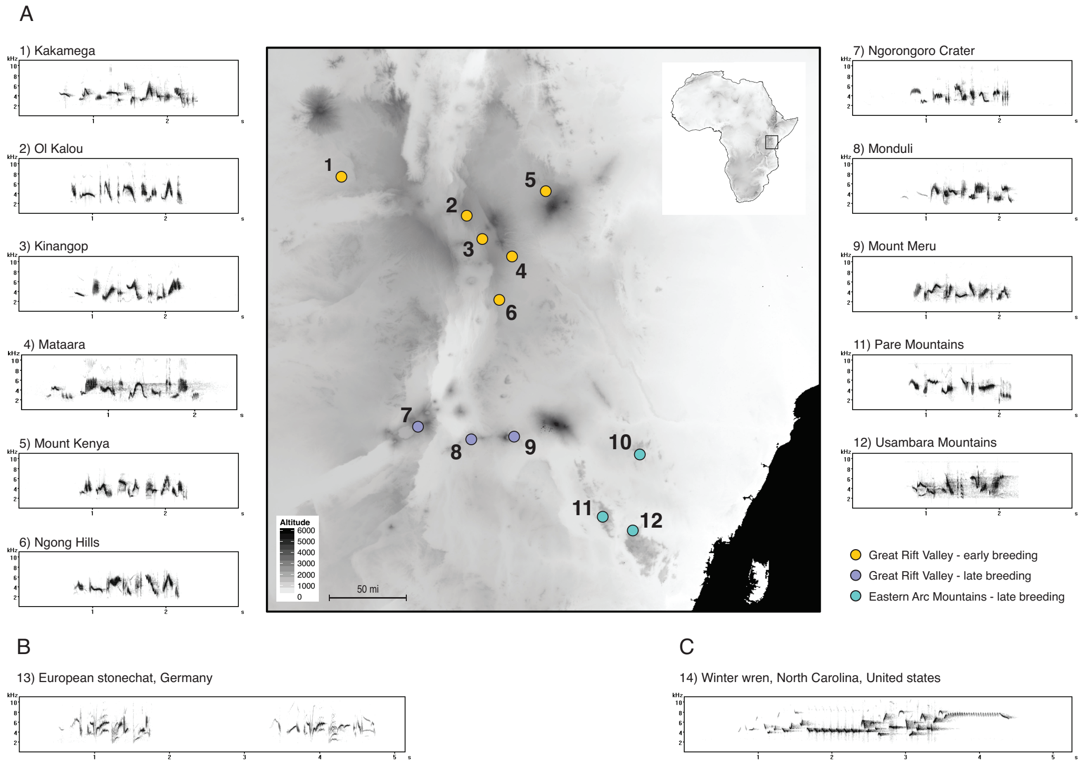

**Fig. 7.10. Exemplary spectrograms and respective geographic locations** for (A) 11 African Stonechat *S. torquata axillaris* populations (Taita Hills excluded), (B) *S. torquata rubicola* (European stonechat) and (C) Winter wren, *Troglodytes hiemalis*, song used as control stimuli during playback experiments.

# Acknowledgements

We would like to thank Peter Grant, Wolfgang Goymann, Fränzi Korner-Nievergelt, Michaela Hau, Wolfgang Forstmeier, Manfred Gahr, Martin Wikelski, Scott La Point, Ulrich Knief, Henrik Kusche, Martin Bulla, James Grecian, Grant Hopcraft, and Davide M Dominoni for valuable discussions about the study, Jack Kiiru, Ann Gathitu, Ireene Madindou, Soi Bernard, Musa Makombe, Wilberforce Okeka, David Ndege and Jan C Habel for assisting in the field, and Gavin J Horsburgh, Andrew P

Krupa, and Rachel Tucker for the support of the genetic analysis. We are also grateful to Yann Gager, Dina Dechmann, Teague O'Mara, Brian Cusack, Adam Wilkins, Theresa Hügel, Ralf Schneider, and Kathryn Elmer for valuable comments on previous versions of this manuscript. We also thank the Museums of Kenya (NMK) and the Tanzania Wildlife Research Institute (TAWIRI) for providing support for fieldwork in Kenya and Tanzania. KGM. was supported by the International Max Planck Research School for Organismal Biology. The study was funded by the German Science foundation (research grant to BH and KGM, grant HE3488/5-1). The funders had no role in study design, data collection and analysis or preparation of the manuscript.

# General Discussion

My doctoral thesis aimed to contribute to our understanding of the evolution in songbirds. What are the ecological and behavioral forces that shape the extraordinary diversity in songbirds? I specifically focused on the role of seasonality, song, and their underlying endocrine regulations as potential drivers leading to population divergence, and eventually speciation.

Evolution requires inter-individual variation, which is subject to natural and sexual selection, and can thereby lead to population divergence. As a first step to understand reproductive isolation mechanisms it is crucial to evaluate the perception of geographic variation in sexually selected traits. Specifically, signaling in the context of resource defense or mate attraction may enhance reproductive isolation (Grant and Grant 2002; Edwards et al. 2005; Price and Sol 2008; Podos 2010). Therefore, I first examined the geographic variation of song and morphological traits in Stonechats, and then tested their behavioral response to phenotypes from different populations. European stonechats discriminated phenotypic traits from different populations during playback and decoy experiments, and this was separately confirmed for African stonechats. Furthermore, both sexes discriminated consistently between stimuli from different populations, responding more strongly to song and morphological traits of local than foreign birds. This finding corresponds well with the local adaptation hypothesis, i.e. birds, which select mates from their natal regions, may gain fitness benefits because their young will more likely express adaptations to local ecological conditions (Marler and Tamura 1962; Nottebohm 1969; Baker 1975; Searcy 1992; Kawecki and Ebert 2004). Interestingly, my results further suggest that song may be indeed the stronger signal for Stonechats than genetically inherited morphological traits. Culturally transmitted song may evolve more quickly, and by promoting reproductive isolation could play a more essential role in incipient speciation (Grant and Grant 2012).

The relationship of local adaptation with associated signaling and female preference may also be affected by a species' life history (Helbig 2003). To gain insights into the geographic variation of song on a larger geographical scale, I therefore studied song traits in relation to the diverse life histories of Stonechats. I focused on a pace of life axis, which includes both demographic characteristics, such as longevity and reproductive output, and seasonal life history traits such as migration or temporary territoriality. In particular, life histories associated with the seasonality of the environment may have striking implications for evolutionary dynamics. My background findings that tropical stonechats follow a slower pace of life than temperate stonechats are concordant with previous studies in Stonechats. Intriguingly, some aspects of song may be indeed linked to different life histories along a latitudinal gradient. In the northern hemisphere, migratory stonechats increased their song activity during the beginning of the breeding season, the time of year when it is most essential to establish territories and attract prospecting females. In sedentary African stonechats, on the other hand, territory establishment and mate attraction only seem to play a minor role for reproduction, since they generally occupy the same territories year-round and form long-term pair bonds. During the short period of the females' maximum fecundity, i.e. nestbuilding, afrotropical males adjusted their song traits to accommodate short-range signaling within close proximity to fertile females. Thereby, they could avoid eavesdropping of other males, and thus prevent extra pair paternity of their mates. Taken together, these findings imply that song of temperate stonechats may be under stronger sexual selection than the sedentary afrotropical populations ((Collins et al. 2009, 2011) but see (Byers 2011)). Seasonal activities such as migration of temperate species may elaborate song as a sexually selected trait because migrants are typically more time constrained for breeding. In migratory populations the strong pressure to find a mate, breed quickly and make the most of the brief breeding season may drive the evolution of more elaborated song than in sedentary populations (Read and Weary 1992; Irwin et al. 2001, 2008; Mountjoy and Leger 2001).

I am also keenly interested in the underlying endocrine regulation of song in birds with diverse life histories. In general, testosterone is known to activate reproductive behaviors, such as song and territoriality (reviewed in (Hau 2007)). Phenotypic variation in testosterone levels seems to follow a latitudinal trend along the pace of life axis. Tropical birds typically lack the dramatic seasonal fluctuations in testosterone shown by males from northern latitudes (Dittami and Gwinner 1985; Reyer et al. 1986; Wingfield et al. 1991; Wikelski et al. 2003a; Onofrei et al. 2004; Garamszegi et al. 2008). In the tropics, testosterone can be involved in regulating song and territorial behavior in year-round territorial birds, although it may remain at low plasma levels throughout the year (Hau et al. 2008). In the stonechats I studied, testosterone was elevated during breeding in all populations regardless of latitude. Compared to other tropical study species, African stonechats experience a rather fluctuating environment with reoccurring patterns of seasonality. They adjust their reproductive timing to such seasonal patterns to optimize their fitness. I suggest that in seasonally breeding tropical species, such as the stonechat, testosterone is elevated during breeding much as in higher latitude birds to amplify territorial behavior and mate guarding during the females' fertile period. Thus, testosterone may facilitate the seasonal timing of reproductive behaviors in tropical species with short, synchronized breeding seasons (Goymann et al. 2004; Goymann and Landys 2011). A previous study found similar seasonal testosterone patterns in Stonechats and proposed that such brief, but pronounced peaks of testosterone may be common in tropical birds, but they may simply be missed if the exact breeding stage of individual birds is not known (Goymann et al. 2006). In light of the seasonal diversity of tropical environments, the pace of life theory and its underlying endocrine control mechanisms should be reconsidered in future studies.

Based on this knowledge, the question arises why birds do not express elevated testosterone throughout the entire year. Testosterone is one important physiological factor proposed to mediate the trade-off between reproduction and survival (Stearns 1989). In particular, it has important pleiotropic effects on resource allocation for mate attraction, territorial behavior, reproductive effort, and associated signal plasticity, while often simultaneously decreasing fitness by suppressing traits such as immune function and parental care. The seasonal adaptation of endocrine regulation may have evolved to circumvent these negative implications of tonically elevated testosterone levels.

In this context, a very astonishing life history trait of some temperate species is the extension of territoriality and associated song into the non-breeding season (Hau et al. 2000). I evaluated the associations of seasonality, song and the endocrine regulation of territoriality in the Black redstart. Using phenotypic engineering, i.e. hormonal manipulation, during simulated territorial intrusions (Ketterson et al. 1996; Hau 2007), I found that during breeding placebo implanted but not testosterone blocked males displayed substantial changes in song structure in response to a territorial intruder. During the non-breeding season, when testosterone levels are naturally low, placebo implanted and testosterone implanted males sang a reduced song, similar to breeding males with blocked testosterone. These results imply that during breeding testosterone seems to facilitate context-dependent changes in song structures. While song activity and structure were regulated by testosterone, non-vocal territorial behaviors were independent of testosterone. The behavioral response to simulated territorial intrusions was similar during breeding and non-breeding. Previous studies observed that in several species outside the breeding season, males defend territories with extended borders, overlapping boundaries or even share these territories with other birds (Wingfield and Monk 1992). Thus, territorial behavior may be only activated by testosterone in a reproductive context, when it is crucial to defend breeding territories (Logan 1992; Schwabl 1992; Wingfield and Monk 1992; Onofrei et al. 2004).

These findings are relevant for my studies of African stonechats, which are well suited for understanding the temporal plasticity of endocrine mechanisms in diversifying seasonal environments. In East Africa, stonechats exhibit considerable differences in timing of breeding between populations on a local scale. In late April 2013, I compared testosterone levels of geographically close populations with asynchronous breeding onsets. Testosterone levels of breeding birds in Kinangop peaked during this time, whereas levels of non-breeding birds from the allochronic population, Mount Meru, were extremely low or could not be detected at all. The highest peak of testosterone levels of this population occurred during their breeding season in late October. Testosterone increase strongly depends on testicular growth previous to breeding. It has been shown that testicular size underlies a rigid circannual cycle and takes about four weeks to fully grow into breeding condition (Helm et al. 2009). These circannual patterns of testicular size and associated testosterone secretion may limit the opportunity for allochronic populations to interbreed, and thus may promote population divergence in African stonechats.

A central aim of the thesis is to contribute to the understanding of the evolutionary dynamics of allochronic populations in African stonechats. I set out to investigate the genetic, song and morphology divergence, and specifically tested for relative contributions of allochronic, spatial and behavioral isolation leading to the present population structure. The results revealed that allochronic populations within the Great Rift Valley, and geographically separated populations from the Eastern Arc Mountains, are genetically differentiated. I examined the environmental connectivity between populations to quantify the relative temporal and spatial contributions to reproductive isolation. Synchronic and allochronic populations along the escarpment of the Great Rift Valley showed similar spatial connectivity, which strongly indicates genetic divergence as the result of allochronic isolation. In contrast, the higher genetic differentiation between populations of the two mountain ranges is rather caused by strong spatial separation. The maximum-likelihood population-effects model, which explained the genetic structure of all twelve populations best, included both temporal and spatial ecological factors. However, the allochronic diversification may have occurred rather recently compared to the more ancient geographic diversification between populations of the two mountain ranges. A preliminary estimate of the divergence time based on the mitochondrial cytochrome b indicates that the genetic divergence between allochronic populations may have commenced at least 100,000 years ago, in contrast to the considerably earlier divergence time between spatially separated populations dating to about 600,000 years ago.

A previous study on captive *Saxicola* species revealed that birds did not modify timing in response to a mate, resulting in decreased reproductive success (Gwinner et al. 1995b; Helm et al. 2009; Bradshaw and Holzapfel 2010). Thus, species such as Stonechats, in which timing of breeding is highly heritable, are expected to have limited phenological plasticity, implying that allochronic populations would experience highly reduced effective gene flow even if they were connected by dispersal (Hendry and Day 2005; Helm et al. 2009). To our knowledge this is the second study providing evidence for incipient allochronic isolation in tetrapods. Such allochronic reproductive isolation mechanisms may drive evolutionary diversification and ultimately speciation, and may be more common than currently recognized.

Remarkably, the genetic structure is strongly concordant with patterns of song and morphology divergence. Concordant with the genetic structure, song traits of all three regions differed from each other, and followed a similar pattern as the genetic structure, whereas morphology traits of allochronic populations from the Great Rift Valley were similar and only differed from the spatially isolated populations of the Eastern Arc Mountains. As mentioned previously, the allochronic diversification may have occurred rather recently compared to the more ancient geographic diversification between populations of the two mountain ranges. The high diversification of song may indicate that the cultural inheritance of song may be a stronger driver for population divergence than genetically inherited morphology, and thus may play a key role in incipient speciation.

In this study, both sexes distinguished local morphological and especially acoustic phenotypes from those of allochronic populations consistently. Females showed preference for song of mates with synchronized reproductive timing. Female preference for familiar vocalizations has been shown in some captive and field experiments by increased copulation-solicitation displays to standardized playback (Baker 1982, 1983; Searcy et al. 2002; Danner et al. 2011), whereas in Stonechats it was measured by approach of the stimulus and associated behaviors. Although the fine acoustic discrimination ability of Stonechats suggests potential behavioral barriers, its implications for geographic isolation are not fully clear, partly depending on song plasticity, and ultimately on the mechanisms involved in song learning. Birdsong is typically learned during a sensitive period early in life. In species that show geographic discrimination, males that subsequently disperse into ranges of other populations would face reduced mating prospects if an acoustic signature of the natal population remains in their repertoire (reviewed in (Podos and Warren 2007). In Stonechats, both sexes had similar sensitivity to incipient behavioral barriers between allochronic populations. The results demonstrate that variation in sexually selected traits may contribute to geographic isolation over relatively short distances, and thereby foster local adaptation.

## Conclusions and future directions

Birds cope with seasonal variation in environmental factors by adaptation of their physiology and life history. When seasonal variation is partly predictable, such adaptations can be based on endogenous circannual rhythms to anticipate reoccurring seasonal change (Helm 2009). The knowledge of local adaptation and adaptive potential of natural populations to seasonal variation is becoming increasingly relevant due to anthropogenic changes in the environment, such as climate change. Although species have responded to climatic changes throughout their evolutionary history, a primary concern for natural populations and their ecosystems is the rapid rate of anthropogenic change. There is already compelling evidence that birds have been affected by recent climate changes (Walther et al. 2002; Parmesan 2006). Most studies report substantial evidence for changes in the phenology of birds, particularly of the timing of migration and of breeding onset; the implications of these responses, however, are not well understood. Many organisms advance the timing of their seasonal activities, thereby adapting to seasonal shifts in their environment (Coppack and Both 2002; Cotton 2003; Both et al. 2004, 2006; Visser et al. 2004). Study populations of African stonechats seem to follow a highly conserved circannual reproductive schedule. I assembled in-depth knowledge of the life history, seasonal life cycle stages, and reproductive physiology focusing on a single population over the past three years. Birds from Kinangop, Kenya, did not attempt to breed outside the breeding season even when environmental conditions were optimal and other species within the same habitat initialized breeding. A long-term study on a geographically close African stonechat population at Lake Nakuru discovered similar findings (Dittami and Gwinner 1985). Neither heavy rains nor high levels of insect abundance were generally associated with any breeding activity outside the breeding season. Interestingly, gonadal development during an extremely dry breeding season was uninhibited, and reproductive hormone levels did not rise with the subsequent belated onset of rain indicating the robust endogenous circannual rhythm of African stonechats (Dittami and Gwinner 1985). Our findings revealed substantial contributions of temporal and spatial isolation to population divergence in African stonechats. Considerable differences in the timing of breeding between populations on a local scale may promote population divergence and ultimately incipient ecological speciation processes.

The reliance on circannual rhythms is fundamental for timing essential annual activities, but may constrain the required fast adaptation to shifting seasonality in an era of climate change. Understanding the endocrine mechanisms by which birds regulate the appropriate timing of breeding as well as the ecology and evolution of seasonal phenotypic traits will be essential for assessing whether populations can adapt their life histories and behaviors to cope with changes in the seasonality of their environment (Visser 2008).

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# Acknowledgements

> *Life is either a daring adventure or nothing at all."*
>
> *Helen Keller*

The past years of my life have been undeniably an adventure for me. One of my truly most desirable dreams since I am a child has become true. And this could only happen because people believed in me, and supported me whenever they could. I feel so much gratitude for every single person who came along with me on this journey, even though or exactly because there have been sometimes tough times together…

First of all, I would like to thank the people who made it possible for me to live my dream…

Barbara, I remember times when there was some doubt lurking around the corner – and maybe it was even reasonable to think twice – I am very grateful that you gave me the freedom to pursue my interests, be independent, and let me explore the African wilderness. I know very well that you always decide with best intentions - You have your heart in the right place! Thank you so much for supporting me especially during my last year in Glasgow! I will always remember us sitting and discussing my projects in tremendous detail for hours, shifting hundreds of little scribbled sheets of paper - trying to organize our minds… I will remember our shared happiness of ginger cookies, coffee, Kember & Jones, chocolate, double fudge chocolate ice cream and even this incredibly stinky cheese topped with a fresh apple slice in our office!

Without the splendid minds of Rosemary and Peter Grant, my DFG grant funding for the African stonechat project would most likely not have become reality. You, your passion for Nature, and forty years of amazing research together just inspire me!

Many people were crucially important for my research, and I would like to thank everybody without exception who contributed to the realization of this thesis. The first thought, which comes to my mind when I think of my PhD, is the time of my fieldwork in Africa and the people who constantly supported me. I am deeply grateful for being so lucky to have such amazing people around me to support me in the field, 24h, every single day… for several months nonstop. Thank you for always being there for me, Ann Gathitu, Jack Gatu Kiiru, Ireene Madindou, Soi Bernard Cherutich, Bea Apfelbeck, and Musa Makombe. You got up at 3 a.m. each morning, drove 13h squeezed between my 'tiny' equipment in the car with me, became professionals in pitching tents in the pitch dark night, you got a driver's license so I didn't need to drive for 13h anymore, we outran wildlife together, you fed me

when I was in a food poisoning delirium for days and so on and on – Honestly, without you this project would not have been possible, and I would not be where I am today!

Many more names cross my mind straightaway, and to name just a few – Asante sana to Jack Mabuti, Jan Christian Habel, Dennis Otieno and his family, all the Friends of Kinangop Plateau FOKP, Nature Kenya, Wilberforce Okeka, Isaac, David Njihia, and all the other field assistants, guarding wildlife rangers, and amazingly hospitable families! I have never felt so welcome! Karibu sana! My field sites were not only based in Africa but also in Germany. Nonetheless, it was as much success and fun since Heiner Flinks just made everything possible and shared his incredible knowledge of Stonechats with me! Dankeschön, Heiner! I also spent a fantastic time with my – I must admit – favorite colleagues Bea Apfelbeck and Sarah Kiefer in Bavaria pursuing the studies on Black redstarts.

All this fieldwork would not have been a success without the tremendous support from the National Museums of Kenya NMK with all the very helpful colleagues and friends; for example, Henry Ndithia, Dominic Kimani, Titus Imboma, Kioko Mukua, and Idle Farah, but also the Tanzania Wildlife Research Institute TAWIRI especially the encouragement by Victor Kakengi, and finally the administration and work shop at the University of Konstanz, namely Silvia Berger, Bruno Erne and Georg Heine, respectively.The International Max Planck Research School for Organismal Biology IMPRS has provided me with an excellent PhD career opportunity. Daniel Piechowksi, Mäggi Hieber Ruiz and Evi Fricke, you gave me the feeling that just everything is possible! Thank you so much for all your effort and positive attitude! I could always count on the most valuable advise from my PhD advisory committee. Our discussions and PAC meetings pushed my research to another level! I am deeply grateful that you followed my projects with great interest and enhanced them with your solid work through collaborations! Thank you so much, Barbara Helm, Wolfgang Goymann, Juan Carlos Illera, Rosemary Grant, Manfred Gahr, and – even though not officially on paper – but also thanks to you, Ela Hau, since you play an equally important role for me!

I have met so many lovely colleagues and excellent scientists at the Max Planck Institute with whom I had fruitful discussions, accurate advise, great collaborations, joyful retreats, and splendid laughter together. Just to name a few - Yann Gager, Ulrich Knief, Mariëlle van Toor, Dina Dechmann, Catarina Miranda, David Santos, Susanne Seltmann, Riek van Noordwijk, Bart Kranstauber, Martin Bulla, René Quispe Valdés, Camila Villavicencio, Yimen Araya, Marina Lehmann, Antje Girndt, Jaap van Schaik, Anne Scharf, Daniel Zuñiga, Lisa Gill, Nina Dehnhard, Stefan Greif, Arnaud Da Silva, Teague O'Mara, Scott LaPoint, Jessica Schnell, Henrik Brumm, Sue Anne Zollinger, Tim Greives, Jesko Partecke, Babette Eid, Alain Jacot, Wolfgang Forstmeier, and Martin Wikelski and so many more…

My stay in Sheffield was an important step for my academic career. Thank you for providing such a well-organized genetic analysis lab and sharing fundamental knowledge in population genetics with me, most importantly Deborah Dawson, Gavin Horsburgh, Andy Krupa, and Terry Burke. I have

certainly made friends with many, but I would like to name my 'BFShefs', Rachel Williams and Rachel Tucker, for becoming such close friends to me!

I spent the last part of my PhD in Glasgow. Planning to come for three weeks and staying for more than a year already tells a lot on how influential – in a very positive way –my colleagues are! Among others I would like to thank Paul Johnson, Jason Matthiopoulos, James Grecian, Grant Hopcraft, Jana Jeglinksi, Iain Malzer, and Jim Caryl for the great time and very impressive discussions!

As most of you know, I am constantly on the move, migrating from Radolfzell to Seewiesen, Reichenau, Sheffield, and from Glasgow to Berlin, Kranenburg, and between many places in Kenya, and Tanzania… In every speck of land there was something I could call 'my home' and lucky me, it was always together with marvelous people - among others Ulrich Knief, María Moirón, Sylvia Rohrer, Dina Dechmann, Heiner Flinks, Jessica Berkes, Lara Bousardt, Tobi Thurau, Ann Gathitu, Michael Curran, Michael Eisenring, Lawrence Wagura, Rachel Williams, Rachel Tucker, Issie Winney, Aaron Comeault, Jana Nešković, Sarah Kiefer, Del Shields, Karine Saline, Adaia Lopez Lopez, Sophie Reichert, Tomaz Spindler & Silvija Delfin – thank you all so much for being there for me! Special thanks to Elke Henkenhaf and Kerstin von Riegen who always did there best during my stay at the MPIO guesthouses – including getting my favorite apartment or room! I am most grateful to my friends at the Reichenau! The place and time we could share together will always be a very fond memory for me - It was just an amazing experience! Dankeschön, Brynia – in my heart you are always with me – Sylvia and Paola Rohrer, Mely Pilz, Max Kapherr, Ale Buttaro, and Davide Dominoni.

Davide, during our PhD we experienced so many exceptional moments, thoughts and emotions together… Our time together shaped me, and I am deeply grateful for this…

Del Shields, I am deeply grateful for your endless effort, the design of this beautiful African stonechat, Ndaracaini, and your positive words and laughter during the last months of my PhD. You are a true friend to me! I would also like to thank my long-term friends especially Melanie Laskowski, Romy Bergmann, Mariam Honarmand – who always inspired me with her tremendously positive thinking - Iris Adam, Conny Bartsch, Silke Voigt-Heucke, Silke Kipper for being so patient with my persistent absence, but extraordinary happiness when we see each other. The same is true for Sarah Kiefer and Gesine Heinrich, but with special thanks for helping me with the very demanding task translating my PhD summary into German, and also including certainly her friend Mara Beck. Jana Nešković, I cannot express how special you are to me! Your love is present wherever I am. I will be always there for you and your family... Just the thought of Malú makes me smile. I can't deny that you are just instant happiness for me! I will always love you.

I could go on and on… but Yann - my toughest reviewer of my mansucripts -would now say 'Cut the crap, Kim!' Just one more, even the most important acknowledgement I dedicate to my parents… I would not be who I am today with all this love and constant support of you! You are always there for me and believe in me. Ich danke Euch von Herzen dafür…

# Author contributions

CHAPTER 1

BEHAVIORAL RESPONSE OF A MIGRATORY SONGBIRD TO GEOGRAPHIC VARIATION IN SONG AND MORPHOLOGY

**Mortega KG, Flinks H, Helm B. 2014 Frontiers in Zoology 11:85**

Conceived and designed the experiments: KGM BH. Performed the experiments: KGM HF. Analyzed the data: KGM. Wrote the paper: BA KGM WG SiK. All authors read and approved the final manuscript.

CHAPTER 2

ASSOCIATED AND DISASSOCIATED PATTERNS IN HORMONES, SONG, BEHAVIOR AND BRAIN RECEPTOR EXPRESSION BETWEEN LIFE-CYCLE STAGES IN MALE BLACK REDSTARTS, *PHOENICURUS OCHRUROS*

**Apfelbeck B, Mortega KG, Kiefer S, Kipper S, Michiel Vellema M, Villavicencio CP, Gahr M, Goymann W. 2013 General and Comparative Endocrinology 184 93–102**

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CHAPTER 3

LIFE-HISTORY AND HORMONAL CONTROL OF AGGRESSION IN BLACK REDSTARTS: BLOCKING TESTOSTERONE DOES NOT DECREASE TERRITORIAL AGGRESSION, BUT CHANGES THE EMPHASIS OF VOCAL BEHAVIORS DURING SIMULATED TERRITORIAL INTRUSIONS

**Apfelbeck B, Mortega KG, Kiefer S, Kipper S, Goymann W. 2013 Frontiers in Zoology, 10:8**

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## CHAPTER 4

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## CHAPTER 5

### DIFFERENT PACE OF LIFE, BUT SIMILAR TESTOSTERONE LEVELS AND BREEDING BEHAVIOR IN TEMPERATE AND AFROTROPICAL STONECHATS

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## CHAPTER 6

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# List of publications

**2015**

Greives TJ, Kingma SA, Kranstauber B, **Mortega KG**, Wikelski M, van Oers K, Mateman C, Ferguson GA, Beltrami G, Hau M. 2014. Costs of sleeping in: circadian rhythms influence cuckoldry risk in a songbird. *Functional Ecology. in press.*

**Mortega KG**, Horsburgh GJ, Illera JC, Dawson DA. 2015. Characterization of microsatellite markers for *Saxicola* species. *Conservation Genet* Resour Volume 7, Issue 1, pp 273-278.

**2014**

**Mortega KG**, Flinks H, Helm B. 2014. Behavioural response of a migratory songbird to geographic variation of song and morphology. *Front Zool 11:185*

**2013**

Apfelbeck B, **Mortega KG**, Kiefer S, Kipper S, Goymann W. 2013. Life-history and hormonal control of aggression in black redstarts: Blocking testosterone does not decrease territorial aggression, but changes the emphasis of vocal behaviours during simulated territorial intrusions. *Front Zool* 10:8.

Apfelbeck B, **Mortega KG**, Kiefer S, Kipper S, Vellema M, Villavicencio CP, Gahr M, Goymann W. 2013. Associated and disassociated patterns in hormones, song, behavior and brain receptor expression between life-cycle stages in male black redstarts, *Phoenicurus ochruros*. *Gen Comp Endocr* 184: 93-102.

**2012**

Apfelbeck B*, Kiefer S*, **Mortega KG***, Goymann W, Kipper S. 2012. Testosterone affects song modulation during simulated territorial intrusions in male Black Redstarts (*Phoenicurus ochruros*). *PLoS ONE* 7: e5200

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Freie Universität Berlin

Responsibility Profile

- Supervision of the bachelor course »Einführung in die Verhaltensbiologie«
- Conception, planning and coordination of multiple experiments, teaching
- Animal care taking
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**Participation in Research Projects**

- Research project on Killer whales, *Orcinus orca*, Tenerife, Canary Islands, Spain
- Research project on Common nightingales, *Luscinia megarhynchos*, in Kenya and Tanzania

**Student Project** Oct 2007
» Importance of the Enrichment for Common nightingales, Luscinia megarhynchos, in captivity, Influences on Behavior «

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| **Related Experience** | May 2008 – July 2010 | Referee at the Hochbegabtenförderung e.V. |
| Responsibility Profile | Promotion for intellectually gifted children in Biology and Chemistry | |

## Talks & Presentations at Scientific Meetings

- Science Slam at the Grand Challenges Symposium on Scientific communication, Max Planck Institute for Ornithology, Seewiesen, Germany (September 2015)
- Partitioning the effects of allochronic and geographic reproductive isolation in an African songbird – 10th International Conference on Behaviour, Physiology and Genetics of Wildlife, Berlin, Germany (September 2015)
- Partitioning the effects of allochronic and geographic reproductive isolation in an African songbird – 10th Conference of the European Ornithologists' Union, Badajoz, Spain (August 2015)
- Allochronic and geographic reproductive isolation in an African songbird – 48th Population Genetics Group, Sheffield, UK (January 2015)
- Allochronic and geographic reproductive isolation in an African songbird – 26th International Ornithological Congress, Tokyo, Japan (August 2014)
- Allochronic and geographic reproductive isolation in an African songbird – Evolutionary Analysis Meeting, BAHCM, Glasgow, UK (March 2014)
- Responsiveness to geographic variation of song and morphology in a migratory songbird – Grand Challenges in Communication in Social Communities, Max Planck Institute for Ornithology, Seewiesen, Germany (September 2013)
- Allochronic and geographic reproductive isolation in an African songbird – National Museums of Kenya, Nairobi, Kenya (March 2013)
- Seasonal changes in testosterone and its effects on singing behaviour and territorial aggression in the Black redstart, *Phoenicurus ochruros* – 2nd Junior Researcher Conference of the German Ornithologists' Society, MPI for Ornithology, Seewiesen, Germany (November 2011)
- Seasonal changes in testosterone and its effects on singing behaviour and territorial aggression in the Black redstart, *Phoenicurus ochruros* – 8th Conference of the European Ornithologists' Union, Riga, Latvia (August 2011)
- Seasonal patterns of singing in relation to breeding in the Common nightingale, *Luscinia megarhynchos* – 5th European Conference on Behavioural Biology, Ferrara, Italy (July 2010)
- Seasonal patterns of singing in relation to breeding in the Common nightingale, *Luscinia megarhynchos* – 13th Junior Researcher Conference of Ethology, MPI for Ornithology, Seewiesen, Germany (November 2009)
- Seasonal patterns of singing in relation to breeding in the Common nightingale, *Luscinia megarhynchos* – 22nd Meeting of the International Bioacoustics Council (IBAC), Lisbon, Portugal (September 2009)
- How pairing changes singing behavior: A field study on nightingales, Luscinia megarhynchos – 4th topical meeting of the Ethological Society, Göttingen, Germany (February 2009)

## Grant Funding & Scholarships

- Travel Grant of the British Ornithologist's Union, 10th Conference of the European Ornithologists' Union, Badajoz, Spain (awarded 2015)
- Travel Grant of the German Ornithologists' Society, 26th International Ornithological Congress (IOC), Tokyo, Japan (awarded 2014) – 1800 €
- Student Research Grant of the International Max Planck Research School for Organismal Biology (IMPRS), Germany (awarded 2014) – 1200 €
- Scholarship of the IMPRS for Organismal Biology, Germany (awarded 2014) – 5700 €
- Student Placement of ERASMUS, University of Glasgow, IBAHCM, Glasgow, Scotland (awarded 2014) – 2000 €
- Research Grant of the NERC Biomolecular Analysis Facility, Sheffield, UK (awarded 2013, substantial contribution) – £ 30,000
- Three-year Research Grant of the German Research Foundation, DFG, Germany, funding of current PhD project (awarded 2011, substantial contribution) – 205,000 €
- Travel Grant of the Equal Opportunity and Women's Affairs, University of Konstanz, 8th Conference of the EOU, Riga, Latvia (awarded 2011) – 800 €
- One-year Scholarship of the IMPRS for Organismal Biology, Germany (awarded 2010) – 20,000 €
- Travel Grant of the Equal Opportunity and Women's Affairs, Free University of Berlin, 5th European Conference on Behavioural Biology (ECBB), Ferrara, Italy (awarded 2010) – 1000 €
- Travel Grant of the Equal Opportunity and Women's Affairs, Free University of Berlin, 22nd Meeting of the International Bioacoustics Council (IBAC), Lisbon, Portugal (awarded 2009) – 1000 €
- One-year Scholarship of the Innovationsfond, Excellence Initiative, Free University of Berlin, Germany (awarded 2009)
- Research Grant of the Equal Opportunity and Women's Affairs, Free University of Berlin, Life histories and hormonal control in Black redstarts, in collaboration with the MPI for Ornithology, Seewiesen, Germany (awarded 2009) – 3000 €
- Travel Grant of the Free University of Berlin, 4th topical Meeting of the Ethological Society – Behaviour and Evolution, Göttingen, Germany (awarded 2009) – 500 €

## Workshops & Summer Schools

- Coping with the challenges of a PhD (Tress & Tress, Munich, Germany)
- Data Visualization (Barret Klein, University of Wisconsin)
- International Summer School in Evolutionary Biology (Basel University & ETH Zurich, Guarda, Switzerland)
- Genomic Sequence Data Handling and Analysis (Jakob Müller, Max Planck Institute for Ornithology, Seewiesen, Germany)
- Scientific Integrity (Roberto Andorno, University of Zurich, Switzerland, 2011)
- Scientific Writing: Introduction (Barbara Helm, Max Planck Institute, Radolfzell, Germany)
- Scientific Writing for Advanced Students (Barbara Helm, Scottish Centre for Ecology and Natural Environment, Scotland)
- Scientific Writing Retreat (Dina Dechmann & Teague O'Mara, Max Planck Institute for Ornithology, Radolfzell, Germany)
- Scientific Writing Lab (Science Craft, Brian Cusack & Adam Wilkins, Berlin, Germany)
- Statistics Module 1: Introduction to R (Fränzi Korner-Nievergelt, Oikostat)
- Statistics Module 2: Linear Models and Linear Mixed Models with R (Fränzi Korner-Nievergelt, Oikostat)
- Statistics Module 3: Generalized Linear Models and Generalized Linear Mixed Models with R (Fränzi Korner-Nievergelt, Oikostat)
- Statistics Module 4: Own Data Workshop (Fränzi Korner-Nievergelt, Oikostat)
- Time Management (Tress & Tress, Munich, Germany)

## Professional Memberships

- British Ornithologist's Union (BOU), since 2015
- Association for the Study of Animal Behaviour, ASAB, since 2010
- German Ornithologists' Society, DO-G, since 2010
- German Zoological Society, DZG, since 2010