Charles University, Faculty of Science, Department of Ecology

Study program: Ecology



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Application of bioacoustic approaches in investigation of bird species interactions and community species richness

Využití bioakustických přístupů pro studium interakcí druhů a druhové bohatosti ptačích společenstev

Doctoral dissertation

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Praha, 2021

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Declaration

I declare that this thesis has not been submitted for the purpose of obtaining the same or any other academic degree earlier or at another institution. My involvement in the research presented in this thesis is expressed through the authorship order of the included publications. Its extent is specified in the statements of contribution from my supervisor and co-authors. All literature sources I used when writing this thesis have been properly cited.

Prague, 18. 10. 2021

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její významná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, 18. 10. 2021

Jana Vokurková

The research presented in this dissertation was financially supported especially by Charles University (GAUK N. 1282214) and the Czech Science Foundation (GAČR P505/11/1617 and P506/10/1155).

Contribution of Jana Vokurková to papers included in her PhD thesis

As a supervisor of this dissertation and a co-author of studies listed below, I declare the contribution of the author Jana Vokurková as given separately for each paper.

October 2021



Ondřej Sedláček

Chapters

Sedláček O, **Vokurková J**, Ferenc M, Nana Djomo E, Albrecht T, Hořák D (2015) A comparison of point counts with a new acoustic sampling method: a case study of a bird community from the montane forests of Mount Cameroon, *Ostrich*, 86(3): 213-220

Jana participated in data analysis, interpretation of results and substantially took part in paper writing.

Vokurková J, Motombi F, Ferenc M, Hořák D, Sedláček O (2018). Seasonality of vocal activity of a bird community in an Afrotropical lowland rain forest. *Journal of Tropical Ecology*, 34(1): 53-64

Jana participated in data collection in the field, data analysis, interpreted the results, and led writing of the manuscript under my supervision.

Contribution of Jana Vokurková to papers included in her PhD thesis

As the senior authors of the publications listed below, we confirm the author contribution of Jana Vokurková as given separately for each paper.

October 2021

Adam Petrusek

Tereza Petrusková

Chapter

Vokurková J, Petrusková T, Reifová R, Kozman A, Mořkovský L, Kipper S, Weiss M, Reif J, Dolata PT, Petrusek A (2013) The causes and evolutionary consequences of mixed singing in two hybridizing songbird species (*Luscinia* spp.). *PLoS ONE* 8(4): e60172

> As the first authorship indicates, Jana had a key role in the bioacoustic part of this study. She substantially participated in data collection in the field, analysed a vast majority of song recordings and interpreted the respective results, and wrote the first draft of the paper.

Appendices

Souriau A, Kohoutová H, Reif J, **Vokurková J**, Petrusek A, Reifová R, Petrusková T (2018) Can mixed singing facilitate coexistence of closely related nightingale species? *Behavioral Ecology*, 29(4): 925–932

> Jana participated on the pilot study in the field, provided feedback on ideas incorporated in the text, and took part in the paper writing and responses to reviewers.

Reif J, Jiran M, Reifová R, **Vokurková J,** Dolata P, Petrusek A, Petrusková T (2015) Interspecific territoriality in two songbird species: potential role of song convergence in male aggressive interactions, *Animal Behaviour*, 104: 131-136

> Jana collected part of the data in the field, shared know-how and closely collaborated with another student member of the team (Martin Jiran) and provided feedback on ideas incorporated in the text.

Souriau A, Geberzahn N, Ivanitskii VV, Marova IM, **Vokurková J**, Reifová R, Reif J, Petrusková T (2019) Singing behind the stage: thrush nightingales produce more variable songs on their wintering grounds. *Behavioral Ecology and Sociobiology* 73, 150

> Jana provided substantial part of Czech recordings, participated in the assessment of element variability, contributed to data interpretation, and provided feedback during the paper writing.

Abstract

Birdsong is one of the most fascinating communication tools in the animal kingdom, whether it is used for attracting a mate or in territory defense. In my dissertation, I present evidence that we can successfully study the role of birdsong in territorial behaviour, its seasonal variability, and ultimately its role in evolutionary processes thanks to modern bioacoustic and ecoacoustic approaches.

By combining the bioacoustic approach with molecular analyses, I demonstrate that in the model system of two closely related passerine species, the heterospecific song convergence in their secondary contact zone is based on cultural transmission, and not on interspecific hybridisation and/or gene introgression.

My dissertation further extends into the tropical zone, which is generally characterized by less pronounced environmental seasonality compared to the temperate zone. The study of potential seasonality in singing activity and the proportion of vocalizing species in this zone therefore requires year-round monitoring, which is possible thanks to the quite recent development of automated recording units (ARU). First, we confirmed that using ARU provides very similar estimates of community composition, species richness, and abundance to traditional field methods (point counts) in the specific conditions of a tropical forest environment.

This allowed me to further study the seasonality in vocal activity patterns of bird species community in the lowland rainforest of Mount Cameroon. I provide interesting evidence that the singing activity and number of species varies significantly in line with the seasonality of rainfall throughout the year and that species turnover in singing activity is highest during the transition periods between the dry and rainy seasons.

My dissertation provides one of the first evidence of year-round distinct seasonality patterns in bird vocal activity in the Afrotropics. I conclude that relatively low-cost, non-invasive, and objective bioacoustic approaches and monitoring are effective in ecological and evolutionary research on bird species in both temperate and tropical ecosystems.

Abstrakt

Ptačí zpěv je jedním z nejvíce fascinujících nástrojů komunikace v živočišné říši, ať už je používán k přilákání partnerů nebo k obraně teritorií. Ve své disertační práci dokládám, že můžeme úspěšně studovat roli ptačího zpěvu v teritoriálním chování, jeho sezónní variabilitu, a nakonec i jeho roli v evolučních procesech díky moderním bioakustickým a ekoakustickým přístupům.

Kombinací bioakustického přístupu s molekulárními analýzami demonstruji, že v modelovém systému dvou blízce příbuzných druhů pěvců je heterospecifická konvergence zpěvů v jejich sekundární kontaktní zóně založena na kulturním přenosu, a nikoli na mezidruhové hybridizaci a/nebo genové introgresi.

Moje disertační práce dále zasahuje do tropického pásma, které se ve srovnání s pásmem mírným obecně vyznačuje méně výraznou sezónností prostředí. Studium potenciální sezonality ve zpěvní aktivitě a podílu vokalizujících druhů ptáků v tropech proto vyžaduje celoroční monitorování, což je možné i díky recentnímu vývoji automatických nahrávacích zařízení (ARU). Nejdříve se nám podařilo potvrdit, že použití nahrávek z ARU poskytuje ve specifických podmínkách prostředí tropického lesa velmi podobné odhady skladby společenstva, druhové bohatosti a početnosti jako tradiční terénní metody bodového sčítání.

To mi umožnilo na Kamerunské hoře dále studovat sezonalitu ve zpěvní aktivitě ptačího společenstva v nížinném deštném pralese. Moje práce přináší zajímavé zjištění, že zpěvní aktivita a počet druhů se v průběhu roku výrazně mění v závislosti na sezonalitě srážek a že obměna vokalizujících druhů je nejvyšší v přechodných obdobích mezi obdobími sucha a deště.

Moje disertační práce tak přináší jedny z prvních poznatků o sezonalitě ve zpěvní aktivitě ptačího společenstva v Afrotropech v průběhu celého roku. V závěru shrnuji, že relativně levné, neinvazivní a objektivní bioakustické přístupy a akustický monitoring jsou účinné v ekologickém a evolučním výzkumu ptačích druhů v temperátních i tropických ekosystémech.

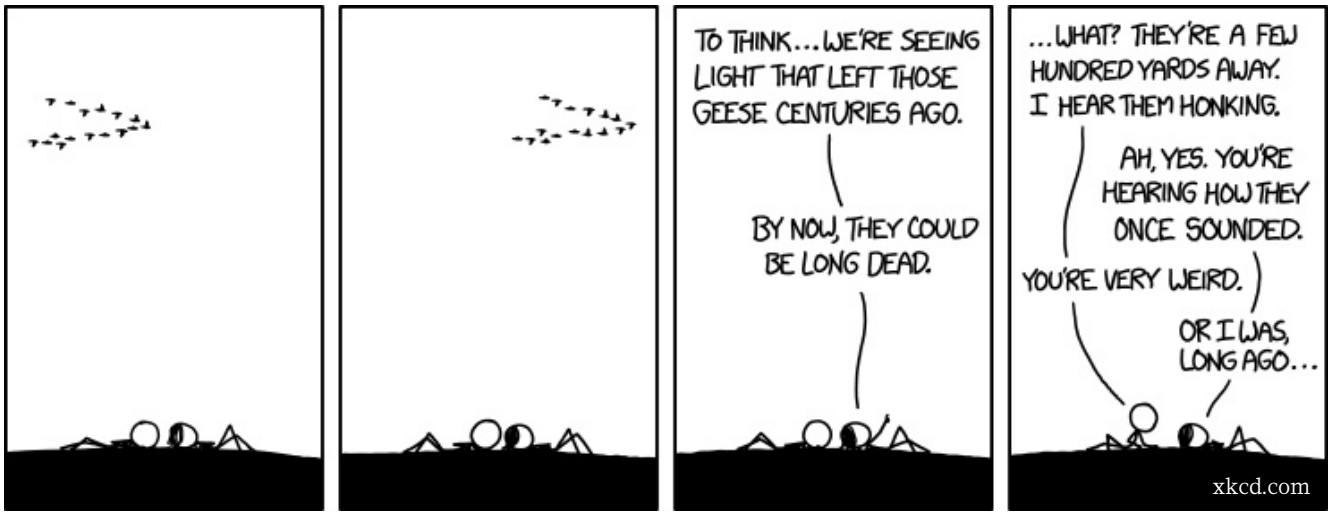
Acknowledgements

First of all, I would like to thank my supervisor Ondřej Sedláček and consultant David Hořák as well as my former supervisors Adam Petrusek and Tereza Petrusková for all their insights and guidance. I have learnt a lot, thank you.

This thesis would never exist without the great help and contribution of many people, including my dear co-authors as well as all my amazing collaborators here in Prague and during my field work in Poland and Cameroon.

I am also deeply indebted to the other scientific teams as well as the reviewers and editors I had a chance to meet or work with during my studies, publication process, and conferences. Not only did I gain valuable experience from them, but I was also exposed to different views on science.

Last but not least, I would like to extend my thanks to my dear friends, classmates, colleagues from "the outside world", and family for the much-needed encouragement and support.



Introduction

Bioacoustics provides us with numerous tools to study a wide variety of phenomena and has the potential to connect many research areas. The versatility and cross-disciplinarity of bioacoustics are what have drawn my attention for a long time. With this introduction and based on the chapters presented and appendices to this thesis, I would like to show that bioacoustics has many fascinating applications in research at the level of individuals, species, and populations, as well as entire animal communities. At the same time, it allows us to observe and study them on various spatial and temporal scales, or along environmental gradients.

Less than a century ago, the pioneers of bioacoustics began to record and publish animal (for the most part bird) sounds primarily for human listening pleasure or to raise public awareness of nature conservation (Krause, 2012). The use of animal sound recordings for scientific reasons came a bit later. In the past, recording animal sounds in the field was a difficult and very expensive activity, but we have made great strides in development over the last 60 years. Sound analysis primarily emerged from military research and music production and was firstly used on birdsong in 1950s (Marler & Slabbekoorn, 2004). Nowadays, we have technology to record and analyse natural sounds across a wide spectrum of frequencies, in any and all environments on Earth and even on very long temporal scales. This gives us new possibilities for research and insights into more ecological rather than behavioural perspectives. The boom of technology has, however, also brought new challenges.

Sound as a carrier of information and its exchange between individuals or species have been studied on many animal taxa and across a wide range of frequencies. As an example, the research spans from the infrasound vibrations of elephants (Herbst et al., 2012) and very low frequency vocalizations of baleen whales (McDonald et al., 2006), through to birds (Mikula et al., 2021), rodents (Bednářová et al., 2013), fish (van Oosterom et al., 2016), dolphins (Herzing, 2015), frogs (Goutte et al., 2018) and the ultrasound of insects (Nakano et al., 2008), bat, or cetacean echolocation (Au, 1997).

Animal sound production has already been studied for quite a long time in the context of animal behaviour, ontogeny, species interactions, and the evolution of communication (e.g., Marler, 1997; Catchpole & Slater, 2008; Tobias et al., 2014), and is more recently being looked at in the contexts of diversity and habitat protection, conservation biology, and wildlife monitoring (e.g., Laiolo, 2010; Obrist et al., 2010; Both & Grant, 2012; Pavan, 2017).

Bioacoustics

Bioacoustics concerns the sounds used by animals for communication and acoustic sensing of the environment. It focuses on behaviour and intraspecific and interspecific interactions, species occurrences, and species-level evolutionary consequences. For the purposes of this Introduction, I will focus only on the vocalizations of birds.

Birdsong is a species-specific trait acting as a central channel of communication between species and individuals (Catchpole & Slater, 2008). It also plays a significant role in mate choice and the maintenance of reproductive barriers (Grant & Grant, 1997a). The divergence in acoustic signals in closely related congeners may be caused by selection for reproductive isolation (Haavie et al., 2004). Divergent character shifts in signals may also be the outcome of interspecific competition for a common resource (Schluter, 2000). On the other hand, signals can converge in secondary contact of species through interspecific gene flow or heterospecific song copying (Alatalo et al., 1990; Grant & Grant, 1997b). In such cases, failing to differentiate between conspecific and heterospecific songs can lead to maladaptive and fitness-decreasing hybridization (Qvarnström et al., 2006).

Recently, modern bioacoustic analyses have enabled us to uncover intraspecific spatial patterns of song structure (e.g., allopatry vs. sympatry of congeners) and to study underlying mechanisms of reproductive isolation and speciation processes in birds (Grant & Grant, 2008, Derégnaucourt, 2010). I have studied such changes in songs on the model of closely related bird species with very complex songs, nightingales in Europe. The project was focused on the common nightingale (*Luscinia megarhynchos*) inhabiting southwest Europe and the thrush nightingale (*Luscinia luscinia*) from the northeast of the continent. The two species diverged already during the Pleistocene and then subsequently came into secondary contact in a recently overlapping area in Eastern and Central Europe (Sorjonen, 1986; Storchová et al., 2010). This makes them a suitable model for studying reproductive isolation mechanisms and the effect of possible hybridisation and introgression on ecology and phenotype in their contact zone.

Song convergence was already observed in these species in the past, and the heterospecific copying of songs seemed to be more common in the thrush nightingale but scarce in the common nightingale (Lille, 1988; Sorjonen, 1986; Becker, 1995). Similarly, some cases of mixed breeding pairs and hybrids have been reported (Becker, 1995), yet only one case was confirmed genetically (Kverek et al., 2008). Thus, in our collaborative project with the Department of Zoology, Charles University we aimed to find out if introgression has an effect on the structure of the song of both species in the contact zone and if heterospecific song copying is affected genetically.

The results are described in **Chapter 1** of this thesis. I used a semi-automated method of cross-correlation to compare the song composition of both species with the existing catalogue of songs of the common nightingale obtained within our collaboration with the team from

Freie Universität in Berlin. We were able to confirm that the phenomenon of „mixed singers" – heterospecific song copying – is widespread in the contact zone of nightingales and is not caused by interspecific hybridisation or introgression but rather by cultural transmission.

These two species of nightingales are morphologically and ecologically very similar (Cramp, 1997). Together with our interesting findings from **Chapter 1**, the logical next step was to investigate the competitive interactions of both species in the contact zone and to try to find out how they react to each other and how it influences their coexistence. We established playback experiments in sympatry to find out how non-vocal aggressive responses as well as vocal territorial responses differ between both species.

The playback experiments revealed that common nightingales reacted more aggressively to the conspecific stimulus than to the heterospecific stimulus. However, thrush nightingales responded to the conspecific as well as to the heterospecific stimulus with the same strength. This seems to be the result of the asymmetric in interspecific copying of songs in thrush nightingales. Mixed singing thus might have an adaptive value in interspecific competition and indeed play a role in interspecific territoriality and species coexistence in their secondary contact zone. The results of the above-mentioned playback experiments are presented in the articles I also co-authored and which are attached as **Appendices 1 and 2**. The Appendices should not be considered as core Chapters of my PhD. thesis here presented.

In intraspecific interactions, birdsong has two main functions – expressing mate quality and defending resources (territoriality) during the breeding season (Catchpole & Slater, 2008). However, migratory passerines (nightingales), like many other species, also sing on their wintering grounds. Only a few studies have investigated why migratory species sing on their wintering grounds, and their conclusions have not yet brought much clarity as to the function of the songs. Winter singing might be caused by the seasonal variation of testosterone levels or by territorial defence behaviour (Kelsey, 1989). Other studies suggested that singing on wintering grounds might improve the fitness of individuals for the next breeding season by song practicing (Sorensen et al., 2016). The song improvement hypothesis could also be the explanation of winter singing in thrush nightingales. The results of our study investigating this hypothesis are presented in **Appendix 3**.

In the temperate zone, the breeding season (and most of the singing activity) of birds is confined to spring and early summer, whereas in most tropical species it is thought to be spread out over the whole year (Stutchbury & Morton, 2001). But while tropical areas host about 80% of bird species richness, there is still little knowledge about the variation of breeding activity among species, functional groups (e.g., feeding guilds), or habitats (Stutchbury & Morton, 2008). Studying year-round vocal activity in tropical birds can shed more light on their breeding habits. However, such extensive temporal and spatial sampling can be challenging, even more so in the

tropics. Beyond the often harsh environmental conditions and expensive logistics, the availability of experts in the field can also be a limitation. The difficulty of intensive year-round investigation of vocal activity in tropical bird communities may be reduced to some degree by the recently developed automated recording systems enabling the monitoring of wildlife over long periods of time (Haselmayer & Quin, 2000, Celis-Murillo et al., 2009, Darras et al., 2018).

As part of wider ongoing ornithological research of in the region, Mount Cameroon National Park was chosen to study year-round vocal activity in tropical birds. The volcanic slopes of Mt. Cameroon provide a rare gradient of tropical environments from lowland rainforests (up to 800 m) through submontane (800-1800 m) and upper montane forests (1800-2500 m), and up to afromontane grasslands above 2500 m a.s.l. (Cable & Cheek 1998). The pristine rainforests and the continuity and extent of elevational gradient offer a great possibility to study not only the species composition and breeding behaviour of tropical birds but also enables the examination of the elevational patterns of species diversity and evolutionary responses of organisms to environmental gradients (Hořák et al., 2019).

To evaluate if new bioacoustic approaches using automated recording unit (ARU) could provide equivalent information to traditional surveys (Bibby et al., 2000a, b) also in the specific environment of tropical forests, we compared samples obtained by traditional point counts with recordings from ARUs in the tropical montane forest environment of Mt. Cameroon. The results of the simultaneous estimates of community composition, species richness, and abundance from field research (point counts) and post-hoc listening of recordings were very similar (**Chapter 2**). This confirmed that the autonomous recording units could be an effective and useful tool for studying bird species assemblages and their ecology in the Afrotropics.

The confirmation that field recordings from autonomous recording units can provide sufficient detail about the vocalizing species of birds in tropical rainforest enabled the further investigation of these species' year-round vocal activity patterns. The seasonality of some environmental conditions (e.g., temperature or day length) is generally less pronounced in tropical than in temperate zones (Moore et al., 2005). However, seasonality in precipitation is an important factor influencing not only the phenology of plants but also the abundance and activity of insects available for birds (Blake & Loiselle, 1991 Ahumada, 2001). Thus, changes in rainfall might influence the breeding behaviour of some feeding guilds. The subject remains quite understudied, yet from available studies it seems that the seasonality patterns of breeding in the tropics are very complex and may substantially vary from none (Hau et al., 2008) to distinct seasonal peaks for some bird species (Williams & Middleton, 2008; Szymanski et al., 2021).

Growing evidence shows that singing activity can be considered a proxy of the breeding activity of birds in the tropics in similar way as in the temperate zone (Chiver et al., 2015; Hill et

al., 2015). Thus, the year-round monitoring of vocal activity might reveal patterns in the breeding behaviour and changes thereto along the environmental gradient. I first focused on seasonality in the vocal activity patterns of the entire bird species community in lowland rainforest. The study presented as **Chapter 3** showed that the singing activity and number of vocalizing species varied significantly in line with the seasonality of rainfall throughout the year and that species turnover was highest during the transition periods between the dry and rainy seasons.

Second, we described seasonal and altitudinal patterns in environmental variables and compared the seasonal vocal activity patterns of bird communities between lowland (350 m a. s. l.), mid-elevation (1100 m a. s. l.), and upper montane rainforest (2200 m a. s. l) on Mt. Cameroon. Clear and distinct seasonality patterns of vocal activity were observed at all three elevations. Seasonality decreases with elevation, likely due to decreasing precipitation as a key environmental variable. The above-mentioned study (**Chapter 3**) is one of the first to quantify the year-round seasonal vocal activity patterns of birds in the tropics at the community level and I intend to publish the results from the elevational gradient comparisons soon (manuscript in prep.).

Further studies of seasonal changes in the singing and breeding activity of individual species, families, or feeding guilds will shed more light on their seasonal breeding preferences and provide valuable information on differences in the evolution of life histories along altitudinal and latitudinal gradients. The effective inventory of bird species communities and the estimation of species abundances along environmental gradients in tropics are needed for the investigation of environmental determinants of bird species richness. The application of new bioacoustic approaches and census methods working with species-specific acoustic signals (Alstrom & Ranft, 2003) seems to be an essential, promising, and non-invasive way to study diversity at large spatial and temporal scales (Sueur et al., 2008; Campos-Cerqueira & Aide, 2017; Sugai et al., 2019) and effectively monitor rare species (Abrahams & Geary, 2020; Metcalf et al., 2021).

Ecoacoustics

Solutions for biodiversity monitoring on large scales, seasonal variation, and habitat spatial distributions as well as relationships to environmental conditions and gradients are the subject of the newly developed field of ecoacoustics. Ecoacoustics has its roots in soundscape ecology (derived from landscape ecology, Pijanowski et al., 2011) and bioacoustics (described above). Researchers defined this interdisciplinary science quite recently (2015). Ecoacoustics combines bioacoustics with the concept of ecology and the study of all sounds and noises that together create the acoustic environment (soundscape), the interactions thereof, and its role in ecological processes (Sueur and Farina, 2015). Soundscape consists of biophony (all sounds produced by organisms), geophony (abiotic natural sounds like rain, wind, waterfalls, etc.), anthropophony, and

technophony produced by humans and machines, respectively. Soundscapes can be considered unique acoustic signatures of the ecosystem (Pieretti et al., 2015) and as such may play a role for example in the increasing interest in habitat degradation over time, the effect of anthropogenic sound on the natural environment, or habitat protection (Farina, 2014). Soundscapes may help answer questions about the roles of animal assemblages in the dynamic of ecosystems (Slabbekoorn, 2004; Burivalova et al., 2018; Pillay et al., 2019) or the human perception of nature (Buxton et al., 2021).

The shift to larger scales of acoustic sampling would not be possible without recent fast advances in technology and declining costs for collecting data via ARUs and storing it. In the last decade, recording quality and the durability of ARUs have improved significantly, such that they are able to persist in harsh environmental conditions; at the same time suitable recording designs balancing technological constraints of power and data storage with temporal demands have been developed (Pieretti et al., 2015; Francomano et al., 2020). Large data volumes and the processing thereof have also brought new challenges. To be able to process and analyse terabytes of acoustic data and to obtain a relevant biological message from it, the ecoacousticians developed various methods for the automated detection of sounds and the species-specific identification of vocalizations.

One group of automated sound recognition approaches uses supervised learning. Generally, the method uses a statistical classifier built from the training data labelled by researchers. Statistical classifiers have been used in individual or multiple-species studies (e.g., Dong et al., 2015; Ruiz-Muñoz et al., 2016; Szymanski et al., 2021), but are less suitable and precise for the automated classification of entire species-rich communities (Campos et al., 2019). The limitation of this approach is the need for large, labelled training datasets. Even with available sound libraries, it is often impossible to obtain sufficient training data and to develop sufficient acoustic recognizers for individual species from real-field recordings of varying quality. This is particularly true for the automated recognition of vocalizations from complex sound and species-rich environments in tropical rainforests with many overlapping or low amplitude signals.

Describing the soundscape is also possible with different methods using acoustic indices. Such indices focus on the overall structure of the soundscape (summarized acoustic energy – intensity or complexity) rather than on target species (Sueur et al., 2014). Since their beginnings in 2013, more than 60 various acoustic indices for terrestrial or underwater environments have been presented so far (Rychtáriková and Vermeir, 2013; Buxton et al., 2018). Some indices or their combinations successfully captured the spatio-temporal dynamics of soundscapes (Rodriguez et al., 2014) or changes in bird species diversity in temperate woodlands (Depraetere et al., 2012). However, it seems that acoustic indices are very sensitive to background noise and its changes or

to the distance and calling rate of target animals (Gasc et al., 2015). For the acoustically complex soundscapes of tropical rainforest with many, varied animal groups overlapping in the same frequency bands (e.g., birds and cicadas), acoustic indices in general do not yet seem to provide accurate estimates of avian species richness and abundances.

The most recent approaches use unsupervised machine learning methods to detect patterns and structures in soundscape recordings without any labelled training data. Such methods can be used for example in the pre-processing phase before the classification of vocalizations in soundscapes (Stowell & Plumbley, 2014). The clustering of automatically detected sonotypes based on their time-frequency attributes can also be used as an alternative or additional approach for the evaluation of environmental acoustic richness from field recordings and their distinctions (Eldridge et al., 2016; Ulloa et al., 2018). Hopefully, in future these methods will also support an increasing amount of passive acoustic monitoring (PAM) initiatives using large networks of ARUs and provide further insights in the ecology of birds (e.g., avian-migrations, Lostanlen et al., 2019) or help with continental-wide biodiversity monitoring and conservation.

Conclusions

To conclude, the recent rapid development of new bioacoustic and ecoacoustic approaches provides a wide spectrum of procedures enabling the study of avian behaviour, reproduction, or territory defense and to dig deeper into the evolutionary mechanisms of avian communication. By using a combination of bioacoustic and genetic analyses on the model system of two passerine species in their secondary contact zone, we showed that interspecific cultural transmission is the major proximate mechanism behind the widespread asymmetric song convergence in nightingale sympatry. The phenomenon of "mixed singers" is not a result of interspecific hybridisation or introgression (**Chapter 1**).

Species-specific bird vocalizations are a reliable proxy and thus the application of acoustic census methods opens new possibilities for effective avian biodiversity assessments and species abundance estimations at large spatial and temporal scales. In the comparison of the traditional census method (point counts) and post-hoc listening to recordings from ARUs we showed that passive acoustic monitoring is also suitable for the dense environment of tropical forests in Africa (**Chapter 2**).

Relatively low-cost, non-invasive, and objective methods of acoustic monitoring seem suitable for the assessment of ecological community characteristics in both temperate and tropical ecosystems and may improve our understanding of how environmental gradients influence animal community patterns. In the year-round study I showed that precipitation is the main factor influencing avian species richness and seasonality in singing activity at the community level in the lowland tropical rainforest of Mt. Cameroon (**Chapter 3**).

Further long-term, multi-species, and multi-taxon studies will bring more insights into ecological dynamics and diversity patterns in tropical ecosystems. Soundscapes may serve as the fingerprints of habitats, and the newly emerging field of ecoacoustics could provide a useful contribution not only to fundamental but also to practical conservation biology, habitat protection, and ecosystem monitoring.

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Chapter 1

Vokurková J, Petrusková T, Reifová R, Kozman A, Mořkovský L, Kipper S, et al. (2013) The causes and evolutionary consequences of mixed singing in two hybridizing songbird species (*Luscinia* spp.). *PLoS ONE* 8, e60172.

OPEN ACCESS Freely available online

PLOS ONE

The Causes and Evolutionary Consequences of Mixed Singing in Two Hybridizing Songbird Species (*Luscinia* spp.)

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Abstract

Bird song plays an important role in the establishment and maintenance of prezygotic reproductive barriers. When two closely related species come into secondary contact, song convergence caused by acquisition of heterospecific songs into the birds' repertoires is often observed. The proximate mechanisms responsible for such mixed singing, and its effect on the speciation process, are poorly understood. We used a combination of genetic and bioacoustic analyses to test whether mixed singing observed in the secondary contact zone of two passerine birds, the Thrush Nightingale (*Luscinia luscinia*) and the Common Nightingale (*L. megarhynchos*), is caused by introgressive hybridization. We analysed song recordings of both species from allopatric and sympatric populations together with genotype data from one mitochondrial and seven nuclear loci. Semi-automated comparisons of our recordings with an extensive catalogue of Common Nightingale song types confirmed that most of the analysed sympatric Thrush Nightingale males were 'mixed singers' that use heterospecific song types in their repertoires. None of these 'mixed singers' possessed any alleles introgressed from the Common Nightingale, suggesting that they were not backcross hybrids. We also analysed songs of five individuals with intermediate phenotype, which were identified as F_1 hybrids between the Thrush Nightingale female and the Common Nightingale male by genetic analysis. Songs of three of these hybrids corresponded to the paternal species (Common Nightingale) but the remaining two sung a mixed song. Our results suggest that although hybridization might increase the tendency for learning songs from both parental species, interspecific cultural transmission is the major proximate mechanism explaining the occurrence of mixed singers among the sympatric Thrush Nightingales. We also provide evidence that mixed singing does not substantially increase the rate of interspecific hybridization and discuss the possible adaptive value of this phenomenon in nightingales.

Citation: Vokurková J, Petrusková T, Reifová R, Kozman A, Mořkovský L, et al. (2013) The Causes and Evolutionary Consequences of Mixed Singing in Two Hybridizing Songbird Species (*Luscinia* spp.). PLoS ONE 8(4): e60172. doi:10.1371/journal.pone.0060172

Editor: Johan J. Bolhuis, Utrecht University, Netherlands

Received September 21, 2012; **Accepted** February 22, 2013; **Published** April 5, 2013

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Funding: The research was supported by grants provided by the Czech Science Foundation (P506/10/1155) to RR, the Grant Agency of the Charles University (632712) to LM, and other funding sources of the Charles University (SVV 267201 and 267204). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Understanding the evolution of reproductive barriers preventing gene flow between incipient species is crucial for understanding the speciation process. Sexually selected traits in general, and those that are culturally transmitted in particular, are considered to play an important role in the origin of prezygotic reproductive isolation [1]. Bird song is one of these traits and its role in the establishment and maintenance of prezygotic barriers in birds seems to be crucial [2–6].

Depending on the type of interspecific interactions, sympatrically occurring species (including closely related species coming into secondary contact) can show divergence or convergence in vocalization patterns, as well as in other key characteristics of species recognition [7]. These may diverge as a result of natural selection, in order to avoid maladaptive hybridization [8] or to reduce interspecific competition [7,9,10]. On the other hand, species may also converge in some features; this is often observed in bird songs (e.g., [6,11]). The mechanisms leading to song convergence may include (1) cross-species song learning [3,12,13], (2) ecological adaptation to the local environment [14–17], and (3) genetic introgression [18–20]. Such convergence can be followed by broad heterospecific pairing, resulting in increased interspecific hybridization and mixing of species gene pools [21–23].

Song in passerine birds is usually learned through an imprinting-like process, although a genetic component of song inheritance has also been described in some species [5,24]. A predominantly cultural transmission of song may cause its rapid divergence in allopatry and thus accelerate the speciation process [25–27]. The same property of song can, however, oppose the speciation in sympatry if heterospecific learning leads to song convergence and increased hybridization and introgression.

Since interspecific hybrids in birds usually have lower fitness compared to their parental species due to sterility of heterogametic

females [6] or inferior ecological characteristics [28–30] (but see, e.g., [18,31]), the convergence in mating signals resulting in increased hybridization rate is assumed to be maladaptive [6]. From this point of view, it seems interesting that song convergence occurs frequently in many secondary contact zones of closely related birds [8,12,23,32,33]. This raises the question whether song convergence in sympatry could be adaptive in some respects. One possibility is that song convergence brings an advantage to males while defending their territories against heterospecific males, and thus reduces the costs of interspecific competition [34]. Furthermore, song convergence may be the result of an ecological adaptation to the local acoustic environment [14–17] or may arise as a by-product of selection on another trait involved in signal production [35–38].

Mechanisms underlying song convergence in secondary contact zones have been studied in detail only in few species including Darwin finches [22,39,40], flycatchers *Ficedula* spp. [8,23] and warblers *Hippolais* spp. [20,33]. The results of these studies indicate that several of the mechanisms mentioned above may contribute to this phenomenon in different species; however, the evolutionary consequences, effects on the speciation process, and a potential adaptive value of song convergence remain poorly understood.

In this study, we investigated the mechanisms underlying song convergence in the secondary contact zone between two nightingale species, the Thrush Nightingale (*Luscinia luscinia*) and the Common Nightingale (*Luscinia megarhynchos*). These species diverged approximately 1.8 MY ago [41], and during the Holocene got into secondary contact in Central and Eastern Europe, with present distribution areas overlapping in a contact zone spanning from northeastern Germany to the Black Sea [42]. Despite the overall morphological similarity, the species can be distinguished by subtle differences in several wing feather characteristics, plumage coloration, and body size [43,44]. Both species have similar ecological requirements but partial habitat segregation has been observed in sympatry [45,46]. The divergence in relative bill size, most likely caused by segregation of feeding niches between the species, has also been documented in areas where both species co-occur [47].

The two nightingale species show strong assortative mating in sympatry. Nonetheless, mixed pairs occasionally arise and produce viable F_1 hybrids, which can be recognized according to their intermediate morphological characteristics [48–50]. Morphological as well as genetic studies suggest that approximately 3–5% of nightingales in sympatry are F_1 hybrids [47,48]. The interspecific hybrids have apparently reduced fitness in comparison with their parental species, particularly due to sterility of F1 females according to Haldane's rule [50,51]. However, the genetic analyses revealed also a small number of backcross hybrids in sympatric populations, although their precise frequency could not be estimated due to a limited number of genetic markers used [47].

The song of both nightingale species belongs to the most complex among songbirds. Nevertheless, there are considerable differences in the song of both species. The repertoire of an average Common Nightingale male consists of some 190 song types [52,53], the Thrush Nightingale repertoires are substantially smaller (up to 42 song types according to [54], and approx. 40–50 song types per male in our samples; Kipper et al., unpubl. data). The latter species sings songs with a longer duration and a lower song rate [44,55], and both species differ in typical song organization (Fig. 1A). As in most oscine passerines, songs in nightingales are acquired through social learning from a model (father or neighbour). Since this learning occurs both during juvenile and adult periods of their lives, nightingales are considered 'open ended learners' (reviewed in [56,57]).

Asymmetric song convergence has been described in areas where the two nightingale species co-occur. A relatively large proportion of Thrush Nightingales living in sympatry include song types from the Common Nightingale in their repertoires [45,48,55,58,59] (Fig. 1A); these birds have been called 'mixed singers'. On the other hand, singing of heterospecific songs is very rare in the Common Nightingale (only one out of 200 studied males was determined as a mixed singer in [55]).

Here we tested whether introgressive hybridization can account for mixed singing in the sympatric Thrush Nightingales. For this purpose, we performed simultaneous analyses of song, phenotype, and multilocus genetic data from sympatric and allopatric populations of both species, including putative hybrid individuals. We used a semi-automated approach for detecting Common Nightingale song types in recordings of both species. This allowed us for the first time to quantify the proportion of heterospecific songs in repertoires of 'mixed singers', as well as to quantitatively compare song composition of interspecific hybrids and pure species.

Methods

Ethics Statement

All necessary permits were obtained for the described field studies. The field work and manipulation with birds was approved by the Local Ethic Committee for Scientific Experiments on Animals in Poznan, Poland (permission no. 27/2008) and by the Ministry of Education, Youth and Sport of the Czech Republic (permission no. 9833/2007-30). Only song recordings were obtained from the German Common Nightingale population.

Field Sampling and Song Recording

The fieldwork was carried out during springs 2007 to 2009 in allopatric and sympatric breeding sites of both species. Allopatric areas were represented by localities in the Czech Republic and Germany for the Common Nightingale and in north-eastern Poland for the Thrush Nightingale. Study sites in sympatry were located in central Poland (see Results and Table S1). All but one sympatric sample were collected in floodplains of Prosna and Warta rivers on the sites with similar densities of both species. One individual with an intermediate phenotype was sampled at the south-western border of sympatry where Common Nightingale dominates. The birds were recorded and captured at the beginning of the breeding season in April and May. They were captured by mist netting, lured into nets (Ecotone, Poland) by playback of commercial recording of either Common Nightingale or Thrush Nightingale [60], depending on the presumed species identity of the respective male. The males were then individually marked by metal and colour rings for later identification. Preliminary species identification of captured birds was based on evaluation of species-specific phenotypic characteristics, including plumage colouration and wing feather measurements [43]. Blood samples for subsequent genetic analyses were collected from each sympatric individual by brachial vein puncture. Samples were stored in pure ethanol until further processing.

The songs were recorded immediately before or within two days after capture (in the same territory) on a digital recorder Marantz PMD660 using a directional Sennheiser ME67 microphone. Altogether, we analysed songs of 41 males including: eleven allopatric Common Nightingales, eight allopatric Thrush Nightingales, nine sympatric Thrush Nightingales, eight sympatric Common Nightingales, and five sympatric males with an intermediate phenotype. Details for individual recordings are given in Table S1. All recordings were obtained during days and

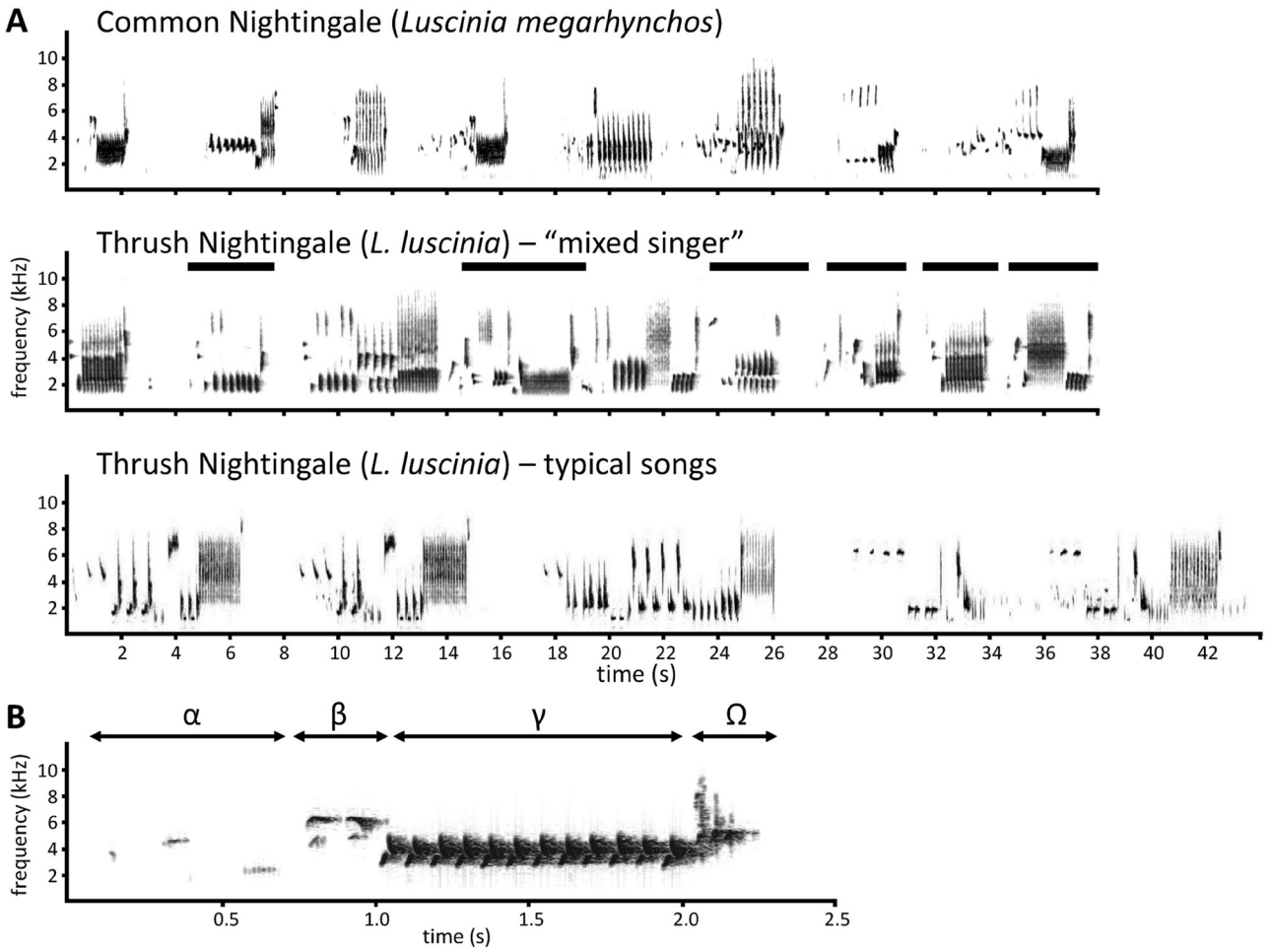


Figure 1. Spectrograms of representative recordings of studied species. Spectrograms of representative recordings of Common Nightingale, Thrush Nightingale "mixed singer", and allopatric Thrush Nightingale (A), with songs characteristic for Common Nightingale in the mixed singer's repertoire indicated by horizontal bars. The first Common Nightingale song from A is enlarged (B) to demonstrate the typical song organization of this species, consisting of alpha, beta, gamma and omega parts (after [52]).
doi:10.1371/journal.pone.0060172.g001

evenings (from 6 am to 9 pm), except for six Common Nightingale males from allopatry that were recorded at night.

Analysis of Songs

Recordings were analysed using the software Avisoft SASLab Pro versions 4.5 to 5 [61]. We analysed approximately 20 minute long recordings of each individual (range 19–23 min; only recordings of two males were shorter, 8 and 14 minutes). The recordings consisted on average of 200 songs per individual in the Common Nightingale (median 204; range 132–265), 148 songs per individual in the Thrush Nightingale (median 148; range 88–206) and 131 songs per individual in males of intermediate phenotype (median 139; range 64–176). Recording length, numbers of analysed songs, results of their assignment to different song type categories (as defined below), and other details are given for each individual in Table S1.

To identify 'mixed singers' among the recorded males, we compared the singing of each male to a catalogue of songs from Common Nightingales based on several German populations. The catalogue consisted of 425 distinct song types and was derived from analyses of nocturnal singing of 50 Common Nightingales (6 years, 3 populations, 533 successive songs per bird equalling about 1 hr of singing; [62]). Each song in our recordings was compared with the catalogue song types and categorized accordingly (see the workflow summarized in Fig. 2). Nightingales are known for their precise song copying and singing, so that despite their large repertoires, song types can be reliably assigned and compared across individuals, populations, and years [53,63].

Though the comparison and categorization of songs can be reliably achieved by visual comparisons of spectrograms, we decided to apply a semi-automated procedure to conduct the categorization as objective as possible. For this we used the spectrogram image cross-correlation analysis in Avisoft SASLab Pro and compared each song with templates of song types included in the catalogue (547 templates, each of 0.5 s duration; some song types consisting of two repetitive phrases were represented by two different templates). These templates were selected to be sections of the 'loudest' part of the respective song type (i.e., the part with the maximum amplitude), which were likely to match other recordings of the same song type.

To facilitate batch cross-correlations, all recordings were first split into files containing single songs. These files were subsequently converted into a format suitable for the analysis with the following settings: sampling frequency conversion 22.05 kHz

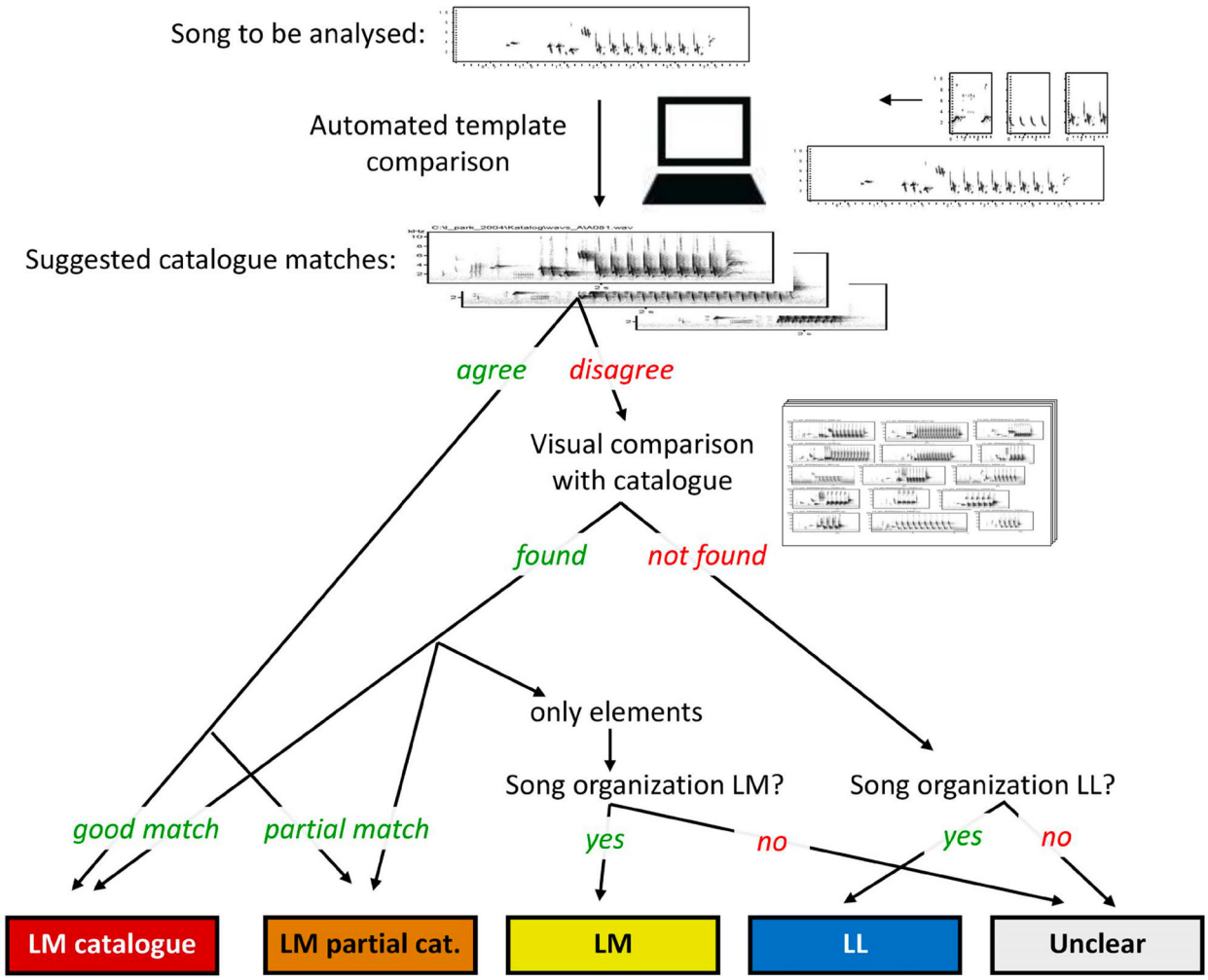


Figure 2. Song analysis workflow: from a recording to category assignment. Visualization of the semi-automated song assignment process comparing each song to be analysed with the catalogue of Common Nightingale song types, and assigning it to one of five song categories (bottom). Visual comparisons were conducted by two people independently of each other. Terms in italics indicate observer decisions (see Methods for details).
doi:10.1371/journal.pone.0060172.g002

(accuracy 256), normalize volume (85%), noise reduction filter (FFT 512, precision 4, threshold −40 db, reduce by 90 db). From each file, a spectrogram file was created (FFT 256, frame size 100, Hamming window, overlap 50%). Matches of the catalogue song templates with each analysed song were evaluated by the function 'Classify.wav or.son files' in Avisoft SASLab Pro, and final visual comparisons were facilitated by an Excel macro that allowed a quick inspection of spectrograms of the best-matching song types from the catalogue. Songs that were not identified by this cross-correlation procedure were re-checked by a visual comparison with the catalogue by two scorers independently from each other and without knowledge of the recording origin.

Each analysed song was assigned to one of five categories reflecting the degree of similarity to the template song types in the catalogue (see examples in Fig. 3): (1) **'LM catalogue'** – songs matching a Common Nightingale catalogue song type exactly or resembling it by at least 95% of the element sequence of the catalogue. (2) **'LM partial catalogue'** – songs similar to a catalogue song type, but differing from it due to absence or difference of some elements, with resemblance of at least 75% of the catalogue song element sequence. (3) **'LM'** – songs that could not be assigned to a catalogue song type, but did show the typical Common Nightingale song organization (alpha-beta-gamma-omega [52]; see Fig. 1B) and recognizable Common Nightingale catalogue gamma parts. (4) **'LL'** – songs that could not be assigned to a catalogue song type, showed typical Thrush Nightingale song organization (beginning with a repeated part, no beta-part, no omega) and did not contain any Common Nightingale catalogue gamma parts. (5) **'Unclear'** – any disputable cases including those of no Common Nightingale song organization, but recognizable gamma parts. Fragmented and poorly recorded song types exceptionally found in our recordings (less than 0.3% of all songs) were excluded from further analyses.

For the final evaluation of song composition of individual birds, each song was assigned to one of the two overall groups, **'Identified'** and **'Not identified'**, depending whether it was identified as a song type typical for the Common Nightingale. The 'Identified' group included the categories 'LM catalogue', 'LM partial catalogue' and 'LM', whereas the 'Not identified' group included categories 'Unclear' and 'LL'. The 'Identified' group thus

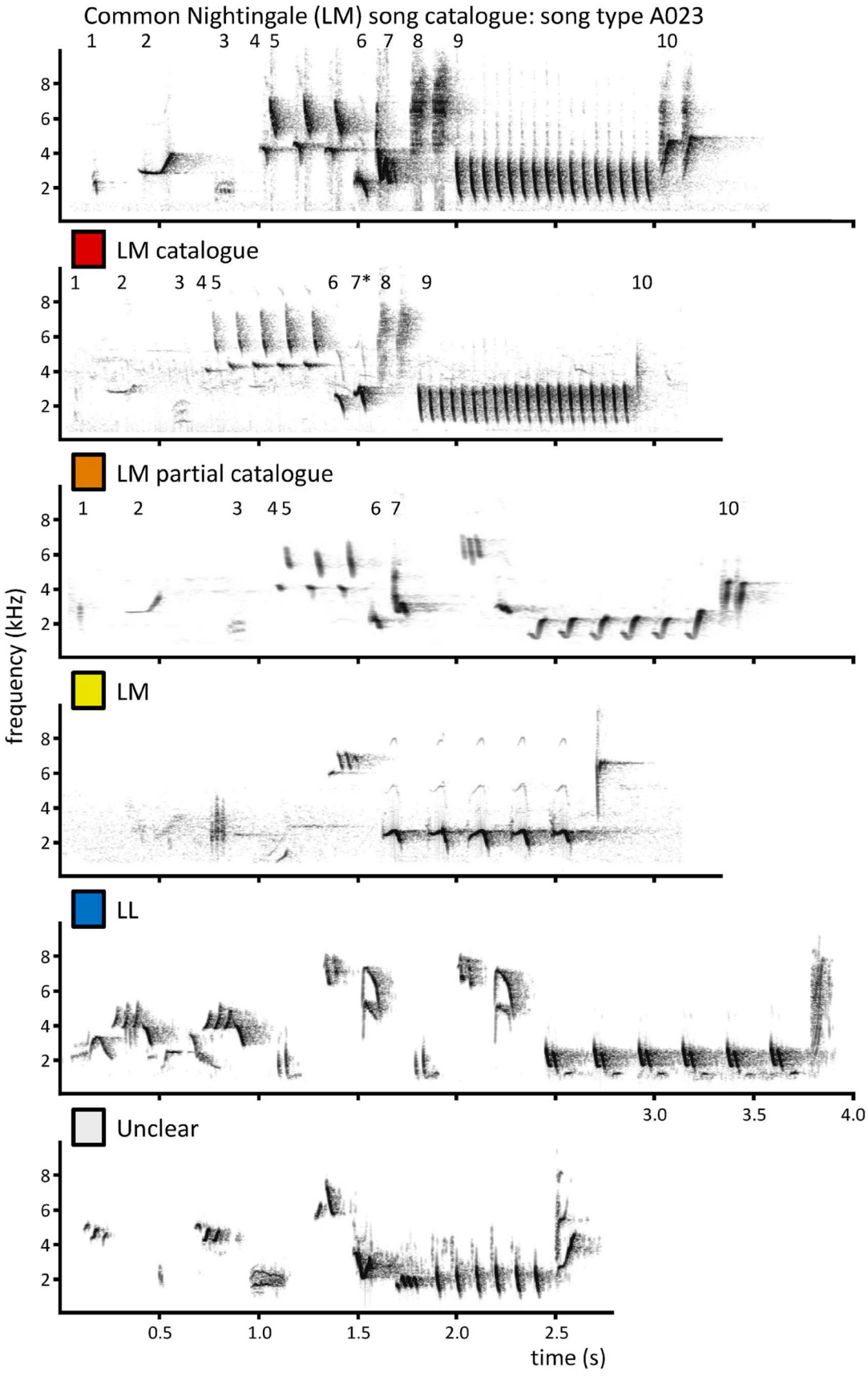

Common Nightingale (LM) song catalogue: song type A023
1 2 3 4 5 6 7 8 9 10
LM catalogue
1 2 3 4 5 6 7* 8 9 10
LM partial catalogue
1 2 3 4 5 6 7 10
LM
LL
Unclear
frequency (kHz)
8
6
4
2
3.0
3.5
4.0
0.5
1.0
1.5
2.0
2.5
time (s)

Figure 3. Examples of song categories, taken from repertoires of sympatric Thrush Nightingales. The Common Nightingale song type included in the catalogue (A023), to which the examples for categories "LM catalogue" and "LM partial catalogue" were assigned, is shown on top. Different elements in the catalogue example and their counterparts in Thrush Nightingale songs are numbered; the asterisk indicates similar but distinct variants. Note that a particular song type is characterized by the order of different elements; the number of their repetitions is variable and does not influence the assignment to particular categories. See Methods and Fig. 2 for definitions of song categories and the workflow of category assignment.
doi:10.1371/journal.pone.0060172.g003

included songs which did not match strictly any song in the catalogue but were most likely of a Common Nightingale origin. The inclusion of this category was thus useful when evaluating the proportion of heterospecific songs sung by Thrush Nightingale mixed singers. Analysing the data with stricter criteria (only 'LM catalogue' and 'LM partial catalogue' included in the 'Identified' group) for evaluation of mixed singing of Thrush Nightingales did not affect the results substantially.

Genetic Analyses

Genomic DNA from the blood sample was isolated by a DNeasy Tissue Kit (Qiagen) and used for PCR amplification of four autosomal and three Z-linked loci, each containing at least one species-informative single nucleotide polymorphism (SNP) (Table 1). The primers for amplification of Z-linked loci are located in conserved exonic regions of the chicken or zebra finch genome so that they amplify intronic sequences [41,64,65]. The autosomal primers amplify transcribed sequences (exons or untranslated regions of mRNA) and were designed according to nightingale transcriptome sequence data (Mořkovský et al., unpublished). Primer sequences and PCR conditions are given in Table S2. One selected species-informative SNP at each locus (except for *ADAMTS6*) was genotyped in all analysed sympatric individuals and 20 control allopatric individuals using ABI PRISM SNaPshot Multiplex Kit (Applied Biosystems). The protocol followed the manufacturer's instructions, used primers and their concentrations in the reaction are provided in Table S3. Genotypes at the locus *ADAMTS6* were obtained by sequencing the whole PCR product.

The obtained SNP data were analysed using the program NewHybrids version 1.1b [66] to estimate the posterior probability that individuals in a sample fall into six pre-defined genotype categories: (1) pure Common Nightingale, (2) pure Thrush Nightingale, (3) F_1 hybrid, (4) F_2 hybrid, (5) first-generation backcross (BC_1) hybrid in the direction of Common Nightingale, and (6) BC_1 hybrid in the direction of Thrush Nightingale. The analysis was performed for allopatric and sympatric individuals pooled, without including prior phenotypic information. The program assumes that the analysed loci are not closely linked to each other. In the absence of physical or genetic map for nightingales, we determined the chromosomal position of each locus in the zebra finch, the only passerine with the known genome (Table 1). All four autosomal loci lie on different chromosomes. The three Z-linked loci are located in different regions of the Z chromosome at least 13.8 Mb apart, a distance roughly corresponding to 20 cM assuming that the recombination rate on the zebra finch Z chromosome is 1.43 cM/Mb [67]. As the studied loci are not closely linked in the zebra finch genome, we can reason that they are unlikely to be all linked in the nightingale genome.

To identify parental species of F_1 hybrids, we sequenced a 525 bp fragment of the maternally inherited mitochondrial gene for NADH dehydrogenase subunit 2 (ND2) using primers and PCR conditions published in [50]. The high quality 193 bp sequence obtained from all hybrid individuals was then compared to previously obtained homologous sequences from 15 individuals of the Common Nightingale and 17 individuals of the Thrush Nightingale [50]. The analysed sequences are sufficiently divergent between the nightingale species, differing by 10 fixed single nucleotide polymorphisms (SNPs), to serve for unambiguous identification of the maternal species.

Statistical Analysis

All statistical analyses were performed in R 2.15.0 [68]. For comparison of song rate between species, we used a non-parametric test (Mann-Whitney U test). To test if there was any difference in song composition between allopatric and sympatric individuals of the same species, we used generalized linear models (GLM, quasibinomial family due to overdispersion, logit link function). Proportions of identified/not identified songs were used as a response variable, and the area of occurrence (sympatry or allopatry) was used as a categorical explanatory variable. The same statistical approach was used for the evaluation of differences in song composition between the sympatric Thrush Nightingale males and F_1 hybrids and Common Nightingale males, respectively.

Table 1. List of seven loci containing species-informative SNPs used for identification of hybrid individuals.

			Species-informative SNPs[2]	
Locus name	**Chromosome[1]**	**Position (Mb)[1]**	*L. megarhynchos*	*L. luscinia*
ADAMTS6	Z	50.7	T (100%)	C (100%)
SPINZ-2	Z	7.5	A (100%)	G (100%)
TG5287	Z	64.5	G (100%)	A (100%)
Lu01	6	18.5	G (95%), T (5%)	T (97.5%), G (2.5%)
Lu03	4	47.1	T (100%)	C (100%)
Lu04	1A	55.0	G (100%)	T (100%)
Lu10	3	64.8	T (80%), C (20%)	C (95%), T (5%)

[1]Position in the zebra finch genome, assembly taeGut3.2.4.
[2]The frequency of alleles occurring in each species was determined in a sample of 20 allopatric individuals of both species.
doi:10.1371/journal.pone.0060172.t001

Results

Analyses of Songs in Sympatric and Allopatric Populations

Altogether, we analysed 6984 songs from 41 males. Of these, 3798 songs were from 19 Common Nightingale males, 2531 songs from 17 Thrush Nightingale males, and 655 songs from 5 males with intermediate phenotype. Thrush Nightingale males sung with a significantly lower rate than Common Nightingale males: on average 7.2 (range 4.2–10.3) songs per minute vs. 9.8 (range 6.1–14.9) songs per minute; Mann–Whitney U test; $N_1 = 17$, $N_2 = 19$, $U = 53$, exact $p = 0.00034$.

One of the Common Nightingale populations from allopatry (Germany) belonged to the populations used for deriving the catalogue. Accordingly, their songs corresponded completely to catalogue song types (Fig. 4A). The other population of males from Common Nightingale allopatry matched the catalogue song types similarly well: on average 98% (range 96–100%; Fig. 4 A,B) of more than 1100 songs of allopatric Common Nightingale males from the Czech Republic were also identified in the catalogue (i.e. category 'LM catalogue' or category 'LM partial catalogue'). Of the remaining 27 songs, 93% were categorized as species-typical Common Nightingale song ('LM'), and only two songs of one male were categorized as either 'Unclear' or 'LL'. Common Nightingale males in sympatry sung on average 83% (range 21–100%; Fig. 4 A, B) of songs identifiable in the catalogue (category 'LM catalogue', 'LM partial catalogue'). All remaining songs were categorized as 'LM'.

There were no 'mixed singers' among the analysed Thrush Nightingale males from allopatry, all their songs were categorized as the song types typical for that species (category 'LL'). In contrast, only one out of nine analysed Thrush Nightingales from the sympatric region seemed not to use any Common Nightingale song types and had apparently a pure Thrush Nightingale repertoire. Seven out of nine males were 'mixed singers', i.e., they used some Common Nightingale song types found in the catalogue and often also additional song types with characteristics typical for the Common Nightingale, together with typical Thrush Nightingale songs in their repertoires. The proportion of these heterospecific songs varied substantially among individuals (on average 62%, range 7–94%), and song categories were represented differently in different individuals (Fig. 4B). Interestingly, the repertoire of the remaining one Thrush Nightingale male contained exclusively Common Nightingale song types. The differences in the proportion of identified heterospecific song types between sympatric and allopatric Thrush Nightingale males were highly significant (GLM, $DF1 = 1$, $DF2 = 15$, $F = 25.94$, $p = 0.00013$), as was the difference in the proportion of Common Nightingale song types in repertoires of sympatric Thrush Nightingale and Common Nightingale males (GLM, $DF1 = 1$, $DF2 = 15$, $F = 14.56$, $p = 0.0016$). In the latter species, no heterospecific song types were recorded in sympatry. Thus, sympatric and allopatric males did not differ significantly in the proportion of Common Nightingale song types (GLM, $DF1 = 1$, $DF2 = 17$, $F = 2.69$, $p = 0.12$).

Two out of five intermediate-phenotype birds were evaluated as 'mixed singers', repertoires of which were dominated by Common Nightingale song types: 86 and 99% respectively (Fig. 4), i.e., higher than in most Thrush Nightingale mixed singers. Three intermediate-phenotype birds apparently used only Common Nightingale song types in their repertoires. Thus, there was a clear trend that intermediate-phenotype males use higher proportion of identified Common Nightingale song types than sympatric Thrush Nightingale males, although marginally non-significant (GLM, $DF1 = 1$, $DF2 = 12$, $F = 4.63$, $p = 0.052$).

Taxon Assignment of Sympatric Nightingales

Using seven species-informative SNP markers we genotyped 20 individuals of each nightingale species from allopatric regions, which should represent pure species, and 22 sympatric individuals with analysed song, including eight with Common Nightingale phenotypic traits, nine of Thrush Nightingale and five of intermediate phenotype. Analysis of the obtained SNP data in NewHybrids confirmed that all allopatric individuals represented pure species (posterior probability >99%). The eight Common Nightingales as well as nine Thrush Nightingales from sympatry were also classified as pure parental species (posterior probability >99%). All five individuals with intermediate phenotype were classified as F_1 hybrids (posterior probability >95%). Analysis in NewHybrids suggested that the studied Thrush Nightingale 'mixed singers' were not BC_1 hybrids. Furthermore, as five of the seven analysed SNPs were species-specific, and none of the 'mixed singers' was heterozygous at any of these loci, it is unlikely that these males were BC_2 or BC_3 hybrids, which are expected to show on average 25% and 12.5% heterozygous loci, respectively.

To determine the species identity of parents of F_1 hybrids, we compared the partial *ND2* sequences from all five analysed hybrid individuals with reference individuals of both parental species. Sequences from all F_1 hybrids randomly chosen in the sympatric population were of Thrush Nightingale origin according to ten species-specific SNPs occurring in this sequence. Accordingly, all F_1 hybrids originated from mating of a Thrush Nightingale female with a Common Nightingale male.

Discussion

Our study brings further evidence that European nightingale species are an excellent model for studying ecological, evolutionary and behavioural consequences of secondary contact and hybridization of closely related bird species. By combining genetics, morphology, and song analyses, we gained a better insight into causes and consequences of the phenomenon of song convergence in their contact zone.

With our semi-automated assigning approach we were able to quantify the occurrence of mixed singers among the Thrush Nightingale males in the area of sympatry with the Common Nightingale. In congruence with former studies [45,48,55], we showed that Thrush Nightingale mixed singers are common in sympatric populations. Frequencies of mixed singers among sympatric Thrush Nightingale males estimated in previous studies were 28% [55], 44% [45], and 56% [48]. In our dataset, this frequency was even higher: seven out of nine sympatric Thrush Nightingale males analysed in this study were mixed singers, one sang only Common Nightingale songs, and only one had a pure Thrush Nightingale repertoire.

The variation in estimates of mixed singer proportions in sympatric populations could be caused not only by differences among studied populations, but also by a higher sensitivity of our method to detect mixed singers. Using this new approach, we quantified the proportion of Common Nightingale song types in the repertoire of individual Thrush Nightingale mixed singers and hybrids for the first time, and showed that it varied considerably among individual birds. Particularly high proportions of Common Nightingale song types were observed in repertoires of the five genetically confirmed F_1 hybrid males: three sung purely Common Nightingale songs, while two used rarely some Thrush Nightingale song types. This might be related to the fact that all hybrid males

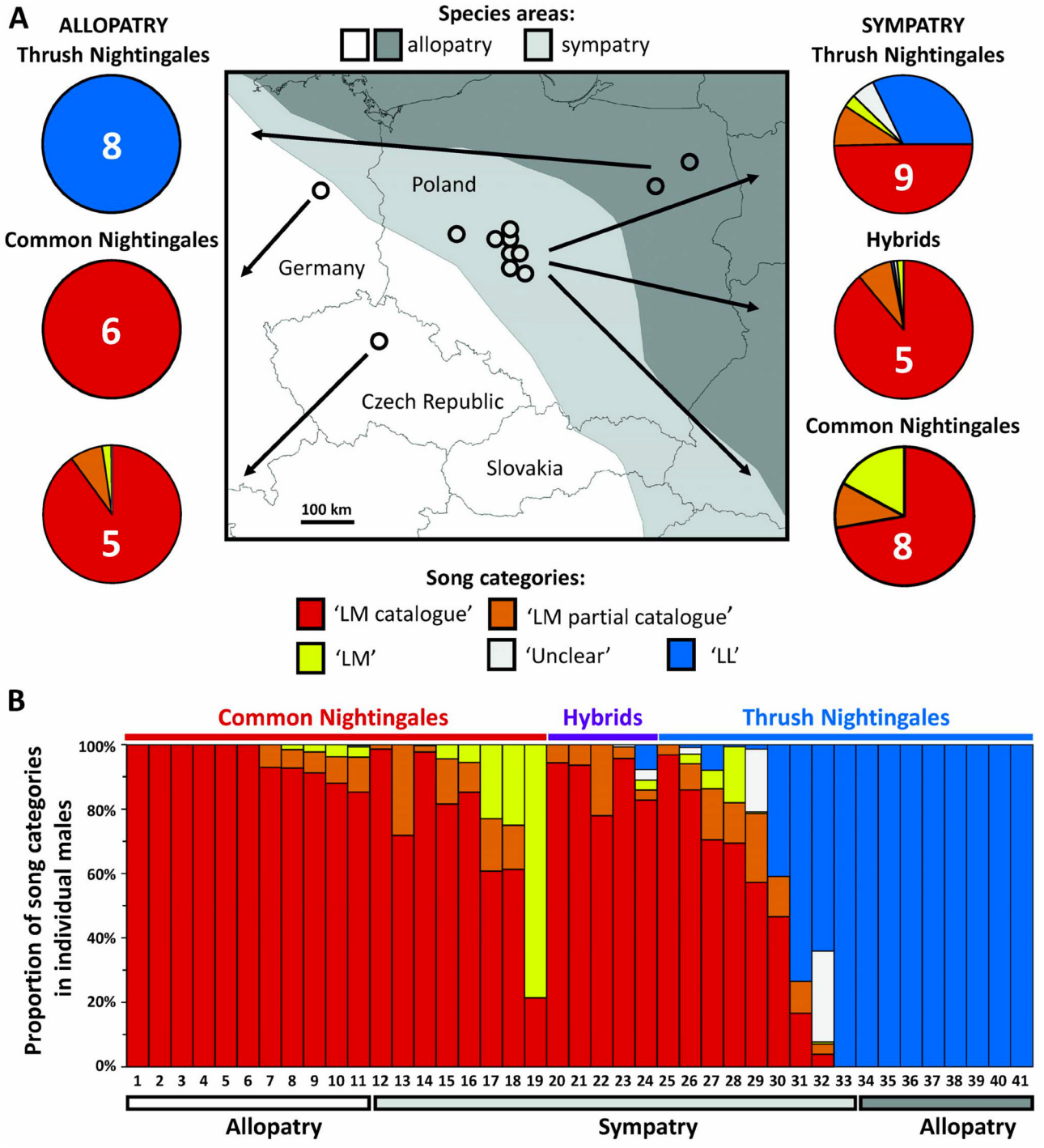


Figure 4. Proportions of song categories in Nightingale recordings from sympatry and allopatry. Proportions of song categories in song samples of allopatric and sympatric Common and Thrush Nightingales. Results averaged for different taxa (pure species and hybrids) and origin (allopatry vs. sympatry) are shown together with a map showing areas of sympatry and allopatry in the studied region (after [47]) and geographic origin of studied males (A). Numbers of analysed males are given in each pie chart; data for allopatric Common Nightingales are shown separately for the German population (the source for the song type catalogue), and for the Czech population. Repertoire compositions of individual analysed birds, ordered according to source region, taxon, and proportions of Common Nightingale song types, are shown in stack bars (B).
doi:10.1371/journal.pone.0060172.g004

were descendants of Common Nightingale fathers, assuming that nightingale juveniles learn songs predominantly from their fathers.

In principle, introgressive hybridization could play an important role in song convergence if the tendency for heterospecific song learning is heritable. The results of our genetic analysis, however, do not support this hypothesis. The Thrush Nightingale mixed singers in our study were not early-generation backcross hybrids and although we cannot rule out that they possess at least some

introgressed loci, we can argue that such old introgression events are unlikely to explain the asymmetrical song convergence in sympatry. Although genetic introgression between the species occurs in both directions (as recently demonstrated in [41]), mixed singers were observed exclusively among sympatric Thrush Nightingales.

Two other suggested ecological mechanisms promoting song convergence, namely (1) adaptations to the local acoustic environment [5,14,16], and (2) adaptive changes in morphological traits affecting song production [35,37,38], are also unlikely to explain the frequent occurrence of mixed singers in the Thrush Nightingale. Such mechanisms are expected to result in quantitative changes in temporal or frequency parameters of the song [14,16,20,69]. They are, however, unlikely to cause acquisition of completely different song types, as happens in nightingales.

Interspecific cultural transmission caused by heterospecific learning, shown to cause mixed singing in *Ficedula* flycatchers [8] or Darwin finches [22,39,40], thus remains the most likely explanation for the frequent occurrence of mixed singers in the sympatric populations of the Thrush Nightingale. The question still remains whether heterospecific learning is adaptive or not, and what causes the asymmetry of heterospecific song copying observed our study species. Song learning experiments in captivity [51] have shown that both species are able to learn the heterospecific song when reared in isolation. Despite that, mixed singing in nature is common in the Thrush Nightingale, but almost absent in the Common Nightingale.

The Thrush Nightingale seems more plastic in song learning from their territorial neighbours. This is supported by higher repertoire similarity among neighbouring males when compared to distant males [54,70]. Such pattern has not been observed for the Common Nightingale, where song repertoires are relatively similar even between distant populations [52,53,70]. Different plasticity in song learning from neighbours could thus partly explain the higher incidence of mixed singing in the Thrush Nightingale. Another possible mechanism likely resulting in strong and asymmetrical cultural transmission includes social pairing of Thrush Nightingale females and Common Nightingale males, with extra-pair offspring sired by Thrush Nightingale males (as observed in flycatchers [71]). Juveniles from such clutches would be genetically pure Thrush Nightingales but learn heterospecific songs. Unfortunately, no data on extra-pair paternity or frequency of mixed pairs are available from the nightingale contact zone.

It has been suggested that song convergence in sympatry may facilitate the development of interspecific territoriality and thus decrease the intensity of interspecific competition [34,72]. Such convergence is likely to be asymmetric; advantageous in the dominant species and maladaptive in the subordinate species [73]. Thrush Nightingales are considered dominant in interspecific competition with Common Nightingales [47], it is thus possible that mixed singers are favoured thanks to more efficient territory defence against heterospecific males. This seems supported by anecdotal observations of males switching from apparently pure Thrush Nightingale to mixed song when counter-singing with a Common Nightingale territorial neighbour [48].

Similar to the song learning in males, female preferences for song also result from a combination of genetic and cultural evolution [74]. Therefore, juvenile females in areas of sympatry may acquire future song preferences by imprinting from both conspecific and heterospecific males. For such females, the more variable song of mixed singers may function as an additive attractant enriching an otherwise species-specific song display. The five hybrids analysed in our study came from crosses between female Thrush Nightingales and male Common Nightingales. Assuming that mate choice in nightingales is strongly based on song characteristics, this indicates that at least some female Thrush Nightingales may have a general preference for more complex Common Nightingale song (similarly as demonstrated for male ornamentation in bright-coloured estrildid finch species [75]). If mixed songs in Thrush Nightingales are more attractive for conspecific females, the proportion of mixed singers among males may increase in time and preferences for mixed singing can eventually establish in a particular population [76]. The high proportion of mixed singers among Thrush Nightingale males in sympatry might be a result of such process.

In contrast to previous studies in *Ficedula* flycatchers [8,23], our results suggest that song convergence in the Thrush Nightingale does not substantially increase the hybridization rate with the Common Nightingale. If mixed singing significantly increased the probability of interspecific hybridization, we would expect elevated hybridization rate between Common Nightingale females and Thrush Nightingale males. Although genetic analyses confirm that hybridization in this direction is possible (Reifová, unpubl. data.), and breeding experiments in captivity proved that F_1 hybrids in both directions are fully viable [51], all hybrids analysed in our study came from the opposite cross. Even if the mixed singers hybridize with heterospecific females slightly more often than males with pure Thrush Nightingale song, this frequency is likely to be very low and does not exceed the frequency of interspecific hybridization in the opposite direction. This could explain why mixed singers are more common in the nightingale then in flycatcher hybrid zone [8].

It is apparent that causes as well as evolutionary consequences of convergence in acoustic signalling substantially vary in different model systems [77–79]. Hybridization and introgression or adaptation to habitat characteristics may play key roles in signal convergence in various vertebrate taxa apart from songbirds, including frogs [80] or non-passerine and suboscine birds [16,19]. Acquisition of signals by learning frequently leads to convergence at intraspecific level, as documented in various groups of mammals and birds [81]. However, vocal convergence of different species resulting from heterospecific learning seems to be a phenomenon particularly important, and best documented, for songbirds. The results of our study suggest that in Thrush Nightingales, mixed singing is not the result of hybridization and introgression. Instead, it may have adaptive value; the fitness loss of mixed singers due to interspecific hybridization is apparently lower than fitness gain due to improved territorial defence and/or increased attractiveness for conspecific females. Further experimental work including playback experiments and testing of female preferences in the nightingale hybrid zone may improve our understanding of selection forces responsible for asymmetrical song convergence in these species.

Supporting Information

Table S1 List of all nightingale males included in song analyses, with information about recordings (date, duration and localities), taxon, number of songs per minute and number of songs in each category per each individual.
(DOC)

Table S2 Primers and PCR conditions for amplification of analysed loci.
(DOC)

Table S3 SNaPshot primers and their concentrations in the reaction.
(DOC)

Acknowledgments

We thank Petr Keil for advice with statistical analysis, Marcin Antczak for obtaining necessary permission for fieldwork in Poland, Jitka Fischerová for help in the field, and two anonymous reviewers for helpful comments on the manuscript.

Author Contributions

Performed the fieldwork: JV JR RR PTD. Designed song analysis workflow: SK MW. Analysed songs: JV SK. Obtained and analysed genetic data: RR AK LM. Conceived and designed the experiments: RR JR AP TP SK. Wrote the paper: JV AP TP RR JR SK.

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Supplementary Table 1. List of all nightingale males included in song analyses, with information about recordings (date, duration and localities), taxon, number of songs per minute and number of songs in each category per each individual.

					Coordinates				Categories						
Number	Taxon	Duration	Date	Locality	N	E	No songs	Song rate	LM catalogue	LM partial catalogue	LM	LL	Unclear	Excluded	% LM songs
Allopatry: Common Nightingales															
1	LM	21:30	April 2008	Berlin (D)	52°29.5'	13°28.3'	150	6.9	150	0	0	0	0	0	100
2	LM	20:00	April 2008	Berlin (D)	52°29.0'	13°29.0'	143	7.2	143	0	0	0	0	0	100
3	LM	20:00	April 2008	Berlin (D)	52°29.1'	13°28.2'	213	10.7	213	0	0	0	0	0	100
4	LM	20:00	May 2008	Berlin (D)	52°29.4'	13°27.7'	217	10.9	217	0	0	0	0	0	100
5	LM	21:30	May 2009	Berlin (D)	52°29.4'	13°27.7'	132	6.1	132	0	0	0	0	0	100
6	LM	20:00	May 2009	Berlin (D)	52°29.4'	13°28.3'	144	7.2	144	0	0	0	0	0	100
7	LM	20:10	May 2009	Předměřice n Jiz. (CZ)	50°14.5'	14°45.4'	199	9.9	183	14	0	0	0	2	100
8	LM	20:35	May 2009	Předměřice n Jiz. (CZ)	50°14.6'	14°46.1'	190	9.2	176	11	3	0	0	0	100
9	LM	23:00	May 2009	Předměřice n Jiz. (CZ)	50°14.9'	14°46.5'	261	11.3	237	17	6	0	0	1	100
10	LM	21:00	May 2009	Předměřice n Jiz. (CZ)	50°15.5'	14°45.9'	212	10.1	184	17	8	0	0	3	100
11	LM	23:00	May 2009	Předměřice n Jiz. (CZ)	50°15.9'	14°46.0'	251	10.9	214	27	8	1	1	0	99
Sympatry: Common Nightingales															
12	LM	20:00	May 2008	Czolnochów (PL)	52°03.0'	17°42.7'	204	10.2	200	3	0	0	0	1	100
13	LM	20:00	May 2008	Czolnochów (PL)	52°02.6'	17°43.5'	161	8.1	115	45	0	0	0	1	100
14	LM	20:21	May 2008	Chocz (PL)	51°58.7'	17°51.1'	259	12.7	252	5	1	0	0	1	100
15	LM	23:00	May 2008	Czolnochów (PL)	52°02.5'	17°43.8'	181	7.9	146	25	8	0	0	2	100
16	LM	14:34	May 2008	Olobok(PL)	51°38.4'	18°04.4'	217	14.9	185	20	12	0	0	0	100
17	LM	20:25	May 2008	Chocz (PL)	51°58.7'	17°51.2'	265	13.0	161	43	61	0	0	0	100
18	LM	20:15	May 2008	Wola Droszewska(PL)	51°37.9'	18°04.8'	212	10.5	130	29	53	0	0	0	100
19	LM	20:00	May 2008	Pogorzelica (PL)	52°08.5'	17°36.1'	187	9.4	40	0	147	0	0	0	100
Sympatry: Interspecific hybrids															
20	HYB	20:00	May 2007	Koscian (PL)	52°08.3'	16°33.6'	176	8.8	166	10	0	0	0	0	100
21	HYB	21:25	May 2008	Pogorzelica (PL)	52°08.3'	17°35.5'	140	6.5	131	9	0	0	0	0	100
22	HYB	20:00	May 2008	Ruda Komorska (PL)	52°07.1'	17°39.9'	136	6.8	106	30	0	0	0	0	100
23	HYB	21:00	May 2008	Raduchów (PL)	51°34.8'	18°09.8'	139	6.6	133	5	0	0	1	0	99
24	HYB	8:11	May 2009	Prusinów (PL)	52°02.9'	17°42.2'	64	7.8	53	2	2	5	2	0	89

Supplementary Table 1 (continued)

Number	Taxon	Duration	Date	Locality	Coordinates N	Coordinates E	No songs	Song rate	Categories: LM catalogue	Categories: LM partial catalogue	Categories: LM	Categories: LL	Categories: Unclear	Categories: Excluded	% LM songs
Sympatry: Thrush Nightingales															
25	LL	20:10	May 2008	Przystajnia (PL)	51°35.1'	18°10.4'	158	7.8	151	5	0	0	0	2	100
26	LL	22:00	May 2009	Czolnochów (PL)	52°02.5'	17°43.6'	200	9.1	172	16	6	2	4	0	97
27	LL	19:36	May 2008	Prusinów (PL)	52°03.0'	17°42.6'	177	9.0	124	28	10	14	0	1	92
28	LL	21:00	May 2009	Chocz site 1 (PL)	51°58.1'	17°52.3'	148	7.0	100	18	25	1	0	4	99
29	LL	20:00	May 2008	Kwileň (PL)	51°59.0'	17°50.6'	206	10.3	118	44	1	3	40	0	79
30	LL	21:00	May 2009	Chocz site 2 (PL)	51°58.7'	17°51.2'	88	4.2	41	11	0	36	0	0	59
31	LL	20:05	May 2008	Chocz (PL)	51°58.3'	17°51.9'	151	7.5	25	15	0	111	0	0	26
32	LL	20:05	May 2008	Pogorzelica (PL)	52°08.5'	17°35.9'	157	7.8	6	5	1	100	44	1	8
33	LL	20:00	May 2008	Czolnochów (PL)	52°02.5'	17°43.8'	119	6.0	0	0	0	119	0	0	0
Allopatry: Thrush Nightingales															
34	LL	20:01	May 2009	Wizna (PL)	53°11.7'	22°22.9'	116	5.8	0	0	0	116	0	0	0
35	LL	20:00	May 2009	Wizna (PL)	53°11.8'	22°22.1'	112	5.6	0	0	0	112	0	0	0
36	LL	19:45	May 2009	Wizna (PL)	53°11.9'	22°22.1'	118	6.0	0	0	0	118	0	0	0
37	LL	20:05	May 2009	Goniadz (PL)	53°34.2'	22°44.8'	180	9.0	0	0	0	180	0	0	0
38	LL	19:59	May 2009	Goniadz (PL)	53°34.3'	22°44.9'	146	7.3	0	0	0	146	0	0	0
39	LL	23:28	May 2009	Wizna (PL)	53°11.7'	22°23.0'	188	8.0	0	0	0	188	0	0	0
40	LL	20:00	May 2009	Wizna (PL)	53°11.8'	22°22.5'	132	6.6	0	0	0	132	0	0	0
41	LL	22:13	May 2009	Wizna (PL)	53°12.0'	22°22.1'	135	6.1	0	0	0	135	0	0	0

Supplementary Table 2. Primers and PCR conditions for amplification of analysed loci.

Locus name	Product size (bp)	Primer sequences (5'-3') Forward primer	Reverse primer	References
ADAMTS6	700	GGAGAGAATGGATTTCTGCC	TGATTCCAGTCTAGGAAACG	[65]
SPINZ-2	1000	ATCATTGTGTCTGCCAGGTG	CCTCAAGACTTCCCCAAAGA	[41, 64]
TG5287	900	GAGTCTTTATCGAGAGCACTGTGA	AATAGCGCCAGAAAGTTTGC	Reifová et al. *in prep.*
Lu01	312	GCACATTTCATGGTCTCCAA	TGAAAAACAGGCTGCAGAAA	This study
Lu03	568	TTCTTGGCATAAATCCATCTGA	ATGCAGTTGCTTCTGCAGTG	This study
Lu04	205	CCATGTCAGATTTAGCATCCA	AGTGGTTTTCAGAGCACAGG	This study
Lu10	272	CAACCAAATGAAAGCTACTGGA	CTCATGCAGACACAACTGTGAA	This study

All PCR reactions of 25 µl volume consisted of $MgCl_2$-free buffer (1x, Promega), $MgCl_2$ (2 mM), primers (0.33 µM), dNTPs (0.2 mM), Taq Polymerase (0.625 unit per reaction), and DNA template (ca 2 ng per reaction). The PCR cycle, identical for all loci, consisted of an initial denaturation step of 2 min at 95 °C, followed by six touchdown cycles with denaturation at 94 °C for 30 s, annealing for 30 s at temperatures gradually decreasing from 60 to 55 °C (by 1 °C per cycle) and amplification step at 72 °C for 60 s, followed by 33 cycles of identical timing with annealing temperature of 55 °C, and a final amplification at 72 °C for 5 min.

Supplementary Table 3. SNaPshot primers and their concentrations in the reaction.

Locus name	SNaPshot primer (5'-3')	Length	Orientation	Concentration in reaction (nM)
TG5287	TGTCAGGTTTAATTTTTTATTTGCAGTAGC	30 bp	F	110
SPINZ-2	AGGGAGTACAAGAATTCATTGTTGTTTTAACAACATTCTG	40 bp	F	55.5
Lu01	$(GACT)_7$CTCGGTCACTGAGTTCTTTCAG	50 bp	F	110
Lu03	$(GACT)_8$AATTACACTGATTATACATTCACC	56 bp	F	222
Lu04	$(GACT)_9$ATCACTACATCAGAGCCTCCTGGGAA	62 bp	F	55.5
Lu10	$(GACT)_{10}$TCCCTTTCTTAATAGGAAATTACTACCT	68 bp	F	55.5

The protocol (including 1. preparation of sample reactions, including a reaction with control DNA and control primer; 2. performing SNaPshot reactions by thermal cycling and post-extension treatment of products; 3. electrophoresing the products on an ABi 3130 Genetic Analyser) followed manufacturer's instructions for the ABI PRISM SNaPshot Multiplex Kit (Applied Biosystems).

Chapter 2

Sedláček O, **Vokurková J**, Ferenc M, Nana Djomo E, Albrecht T, Hořák D (2015) A comparison of point counts with a new acoustic sampling method: a case study of a bird community from the montane forests of Mount Cameroon, *Ostrich*, 86, 213–220.

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OSTRICH
ISSN 0030–6525 EISSN 1727-947X
http://dx.doi.org/10.2989/00306525.2015.1049669

A comparison of point counts with a new acoustic sampling method: a case study of a bird community from the montane forests of Mount Cameroon §

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Acoustic signals are frequently used for estimating avian species richness, abundance and community composition. However, sampling by traditional methods of bird surveys is often limited by availability of experienced researchers in the field, especially in the tropics. New bioacoustic approaches offer some solutions to such limitations and provide opportunities for more extensive spatial and temporal sampling. In our study, we compared results of traditional point counts with simultaneous acoustic samples obtained by automated soundscape recording units in the montane forest of Mount Cameroon. We showed that the estimates of species richness, abundance and community composition based on point counts and *post-hoc* laboratory listening to acoustic samples are very similar, especially for a distance limited up to 50 m. Species that were frequently missed during both point counts and listening to acoustic samples were typically those with relatively quiet songs. Abundances were rather underestimated by listening to acoustic samples in the most abundant species, including those occurring in flocks and species with low singing activity. Despite some possible biases, we demonstrated that the method based on listening to acoustic samples is relatively effective and offers a useful alternative approach for surveying Afromontane bird communities.

Keywords: abundance, automatic recording units, montane forest, point count, species richness, species turnover

Introduction

Point counts with distance sampling are the most frequently used method for estimating avian species richness, abundance and community composition (Buckland 2006; Simons et al. 2007). However, field sampling of avifauna by such traditional methods is often limited by the availability of experienced observers (Hobson et al. 2002). The development of alternative sampling approaches during the last decade, including methods based on automatic recordings, brings new opportunities for investigation of bird communities, especially in the tropics (Venier et al. 2011; Celis-Murillo et al. 2012; Sueur et al. 2012). New bioacoustic approaches offer more cost-effective, time-efficient, and spatially and temporally extensive sampling (Haselmayer and Quinn 2000; Hobson et al. 2002; Celis-Murillo et al. 2009). Devices suitable for acoustic sampling of bird communities produce soundscape recordings, i.e. sample all sounds produced in a given environment including background noise (Sueur et al. 2012). Advantages of using automatic soundscape recording include the possibility to schedule recording over multiple time periods with a single visit, the production of permanent, verifiable records, the control for observer variability, and the possibility to employ field assistants with limited bird identification and survey skills (Hutto and Stutzman 2009; Blumstein et al. 2011; Rempel et al. 2013). Permanent recordings also could be made easily available for the use of other researchers and for future comparisons.

However, before automatic soundscape recording is accepted as a rigorous technique and applied more generally, there is a need to evaluate its performance, compare outcomes with those of conventional census methods and identify possible biases (Hobson et al. 2002; Acevedo and Villanueva-Rivera 2006; Hutto and Stutzman 2009; Rempel et al. 2013). Recent studies conducted in different habitats in the temperate zone generally revealed that listening to recordings enables detection of a similar number of species as revealed during point counts in the field (Hobson et al. 2002; Rempel et al. 2005; Cellis-Murillo et al. 2009; but see Hutto and Stutzman 2009). Rempel et al. (2013) also showed that data recorded by different microphone systems do not differ much from actual number of bird species detected by observers. The only study from the tropics (Yucatan Peninsula), conducted by Celis-Murillo et al. (2012), also brought promising results; these authors showed that species richness, species composition and detection probabilities of 15 bird species did not differ

§ This paper was presented at the 13th Pan-African Ornithological Congress, Arusha, Tanzania, 14–21 October 2012

between a quadraphonic acoustic recording system and point counts in six structurally different vegetation types, excluding tropical forest. However, performance of acoustic monitoring has never been tested in tropical rainforests and the specific environment of tropical montane forests, especially with regard to distance-related limitations of the method. For example, sound transmission conditions in the tropical forest interior differ from other temperate and drier tropical environments in many aspects, such as structure and density of vegetation, humidity or ambient noise, and can be influenced by species community composition, bird vocal activity and song structure (e.g. Eve 1991; Mathevon et al. 2008).

Our study was situated in pristine montane forest of Mt Cameroon, which is recognised to form part of one of the most important areas of bird endemism in Africa (Fishpool and Evans 2001; BirdLife International 2013). Our general objective was to compare the results of automatic acoustic sampling in estimating alpha, beta and gamma diversity, species composition and abundances of the bird community with simultaneously conducted point counts and to evaluate spatial limits of acoustic sampling. In particular, we aimed to determine if the two survey methods generated similar estimates of (1) the number of species (hereafter 'species richness') at particular census points, (2) community similarity among sites, and (3) total abundances of the species in the study area. In addition, we aimed to determine (4) if and how these estimates differ for different sampling extents, and (5) sources of bias for particular species.

Material and methods

Study site

The study was conducted in the interior of the primary upper montane forest of Mt Cameroon National Park, South-West Region, Cameroon. The site was located on the south-western slope of the mountain close to Mann's Spring (4°08.67′ N, 9°07.31′ E) at an altitude of approximately 2 250 m above sea level (asl). Average annual precipitation at this altitude on Mt Cameroon is about 3 000 mm and persistent mist and cloud cover usually occurs. The rainy season lasts from late March to the beginning of November, with a peak during July–September (Payton 1993; Fonge et al. 2005). The montane forest extends from c. 1 600 m up to 2 300 m asl at the study site with small fragments up to 2 500 m asl (Cable and Cheek 1998). The forest at the study site represents a typical upper Afromontane forest with discontinuous canopy, which is 10–15 m high, and a high frequency of large trees (Proctor et al. 2007). Trees carry a heavy load of epiphytes, particularly mosses and ferns. The understory consists of dense, tall herbs and shrubs, about 2 m tall (Thomas 1986). The most abundant tree species in the study area included *Schefflera abyssinica*, *S. manni*, *Prunus africana* and *Podocarpus* spp.

A total of c. 375 bird species have been recorded from the Mt Cameroon area, 61 of which are recorded at c. 2 300 m asl (Bayly and Njie Motombe 2004; our unpublished data), although the latter figure includes montane grassland species and Palearctic migrants. There is a high level of endemism among the avifauna of Mt Cameroon, with 20 species confined to the Cameroon Mountains Endemic Bird Area and two species (Mount Cameroon Francolin *Francolinus camerunensis* and Mount Cameroon Speirops *Speirops melanocephalus*) only found on Mt Cameroon. The majority of these endemics are confined to montane forest (BirdLife International 2013).

Field sampling

Field work was carried out between 9 and 15 November 2011, at the beginning of the dry season. This period corresponds to the breeding season of the majority of montane forest birds of the Cameroon mountains (Serle 1981; Tye 1986). We sampled bird communties using conventional point counts as described by Bibby et al. (2000a). Point counts are a suitable sampling method in closed forest habitats with high canopies, particularly rainforests, and can be used for estimating species abundances (Bibby et al. 2000b). We selected 16 census points spaced 150 m apart. The distance between census points was specified so as to diminish the probability of multiple counting of individuals while remaining logistically tractable for the study. Point counts were performed between 06:00 and 10:00 in the morning. We conducted three 15-minute point counts at each census point (n = 16), resulting in 48 point samples. No census point was sampled twice on the same day. Point counts were conducted by two groups of researchers (two or three persons) led by two researchers who primarily sampled the bird community (OS and DH). OS collected 28 and DH collected 20 samples. Each researcher conducted at least one of the three samples at each census point. We recorded all heard and seen individual birds during point counts. If an individual was both heard and seen, it was considered to be recorded acoustically. Each point count was divided into three 5-minute intervals, and the bird community was sampled separately for each interval. The distance to each bird detected was recorded as either less than 50 m or more than 50 m. A range finder was used to calibrate the 50 m distance at each census point.

Simultaneously to conducting each point count, a 15 min acoustic sample (AS) was obtained using SongMeter recording units (SM2, Wildlife Acoustics, Inc., Concord, MA, USA) with two sensitive omnidirectional microphones (see Sueur et al. 2012 for detailed description of the devices). The devices were mounted on the nearest tree stem at a height of approximately 180 cm and recordings commenced and ended at the same moment as each point count. The sampling frequency of the acoustic samples was 44.1 kHz and the samples were saved in WAV format on 32 GB SDHC cards.

Data processing and analyses

As was done with sample point data, the 48 acoustic samples were each divided into three 5-minute samples using GoldWave 5.67 software (http://www.goldwave.com). These samples were randomly renamed from 1 to 144, so that they could be processed 'blindly'. Samples were studied by OS who listened to the original WAV files using a computer (played in GoldWave) and stereo channel headphones under standardised conditions. The recording was played with a volume approximately simulating real

noise, and each individual bird that was heard was noted to compile a list of species and the number of individuals of each species recorded on each acoustic sample. All detections of individuals were divided into two categories according to the clarity of its vocalisations: clearly audible individuals (approximately up to 50 m) and individuals that were not clearly audible (more than 50 m).

The three 5-minute acoustic samples made during a point count were grouped together and the cumulative species richness for 5-, 10- and 15-minute sampling periods was calculated. For comparison of species richness detected during point counts and listening to acoustic samples we used all 48 original 15-minute samples (three repeats at 16 census points). We treated these samples as independent simply because the aim was to compare the species richness estimated by two methods at the same time. However, community similarity analysis was based on census points (n = 16), where the species composition was grouped for all three visits at a census point. Similarly, species abundances (number of individuals of each species) per census point were estimated as the maximum estimated abundances for particular species recorded during the three visits at a census point. Total abundance of a species for the whole study site was estimated as the sum of these maximum per-point abundances.

To compare species richness and species abundances estimated by point counts and acoustic sampling, we employed basic statistical tools such as paired t-tests and Pearson´s correlations. There was no need to transform the species richness data because it fitted a normal distribution. The similarity of bird communities between all particular census points was estimated using the Sørensen index (Sørensen 1957). All statistical analyses were conducted using R 2.15.0 software (R Development Core Team 2011). Means are presented with standard deviations (SD).

Results

We recorded a total of 30 bird species during point counts. All species, with one exception (the uncommon Black-billed Weaver *Ploceus melanogaster*), were also detected using acoustic sampling. Almost all species recorded during point counts were detected only (18 species) or mostly (more than 73% of individuals in seven species) by vocalisations. Only five species were seen during a point count more often than they were heard (Table 1).

Table 1: A comparison of the two methods (point counts and acousting sampling) used for estimating number of detections for point samples (n = 48) and total abundances at census points (n = 16, three controls) for all bird species. Two distances from observer/recorder were distinguished: within a radius of 50 m (clearly audible individuals in acoustic samples) and at any distance. The % Vocalising column shows the proportion of individuals for a particular species that were detected by vocalisation during point counts in the field for any distance from the observer

Species	Number of detections (n = 48)				Abundance (n = 16)				% Vocalising
	Point count		SongMeter		Point count		SongMeter		Point count
	50 m	All	50 m	All	50 m	All	50 m	All	Total
Andropadus tephrolaemus	48	48	47	47	49	51	34	34	98.5
Apalis cinerea	41	47	38	45	25	39	17	28	96.5
Bradypterus lopezi	37	40	38	40	28	36	23	34	100
Cinnyris reichenowi	47	47	47	47	53	54	37	37	100
Cisticola chubbi	26	36	30	35	17	28	13	21	100
Columba sjostedti	2	2	1	1	2	2	1	1	0
Cossypha isabellae	21	23	15	15	17	18	13	13	93.1
Cyanomitra oritis	10	10	4	4	6	6	3	3	33.3
Dendropicos ellioti	13	14	9	10	7	8	8	9	85.7
Elminia albiventris	19	20	20	20	12	13	14	14	92
Francolinus squamatus	0	4	0	3	0	5	0	3	100
Indicator willcocksi	5	9	5	9	5	8	6	9	100
Kakamega poliothorax	2	2	2	2	1	1	1	1	100
Laniarius atroflavus	42	48	40	48	34	52	19	36	100
Linurgus olivaceus	15	17	20	21	14	14	16	16	100
Muscicapa adusta	15	16	18	18	10	15	15	15	72.7
Onychognathus walleri	6	7	4	6	35	48	11	16	100
Phylloscopus trochilus	1	5	1	2	1	5	1	2	100
Platysteira cyanea	19	40	24	40	17	30	16	25	100
Pogoniulus coryphaeus	21	36	23	32	12	24	14	20	95.5
Psalidoprocne fuliginosa	1	1	1	1	1	1	2	2	0
Pseudoalcippe abyssinica	46	48	46	48	34	45	27	38	100
Pycnonotus barbatus	4	4	4	4	2	2	2	2	100
Saxicola torquata	3	4	3	3	1	2	1	1	100
Serinus burtoni	2	2	2	2	2	2	2	2	100
Speirops melanocephalus	7	7	9	9	11	11	12	12	100
Tauraco macrorhynchus	2	2	0	2	2	3	0	4	0
Turdus pelios	16	16	11	11	11	12	1	1	100
Urolais epichlora	21	30	23	28	18	22	15	17	100
Ploceus melanogaster	1	1	0	0	1	1	0	0	0

The cumulative species richness detected within 50 m of census points during point counts increased from 6.85 ± 2.08 (5 min) to 8.85 ± 2.20 (10 min) and to 10.23 ± 2.46 (15 min) per point sample (n = 48). Almost identical results were obtained for clearly audible birds during acoustic sampling, with species richness increasing from 6.88 ± 2.21 (5 min) to 8.85 ± 2.18 (10 min) and to 10.06 ± 2.28 (15 min) per point sample (Figure 1a). We observed no significant difference in species richness estimated by the two methods for any of the three time intervals (paired t-test, df = 47; 5 min: t = 0.09, p = 0.9; 10 min: t ~ 0, p = 1; 15 min: t = −0.80, p = 0.5). The species richness detected for particular 15-minute samples by the two methods was also significantly correlated (r = 0.82, p < 0.001; Figure 2a). The number of cases above (15) and below (20) the line of direct correlation (slope = 1.0) were similar, suggesting that the two methods produced a comparable amount of inaccuracies in estimation of the species richness.

For birds detected at any distance from the observer, the cumulative number of bird species detected during point counts increased from 8.60 ± 2.01 (5 min) to 10.81 ± 2.18 (10 min) and 12.21 ± 2.47 (15 min) per point sample (n = 48). During acoustic sampling, the cumulative species richness increased from 8.44 ± 2.12 (5 min) to 10.35 ± 1.98 (10 min) and 11.48 ± 2.05 (15 min) per point sample (Figure 1b). We detected significantly higher cumulative species richness during the point counts compared with acoustic sampling after 10 min and 15 min, respectively (paired t-test, df = 47; 10 min: t = −2.25, p = 0.03; 15 min:

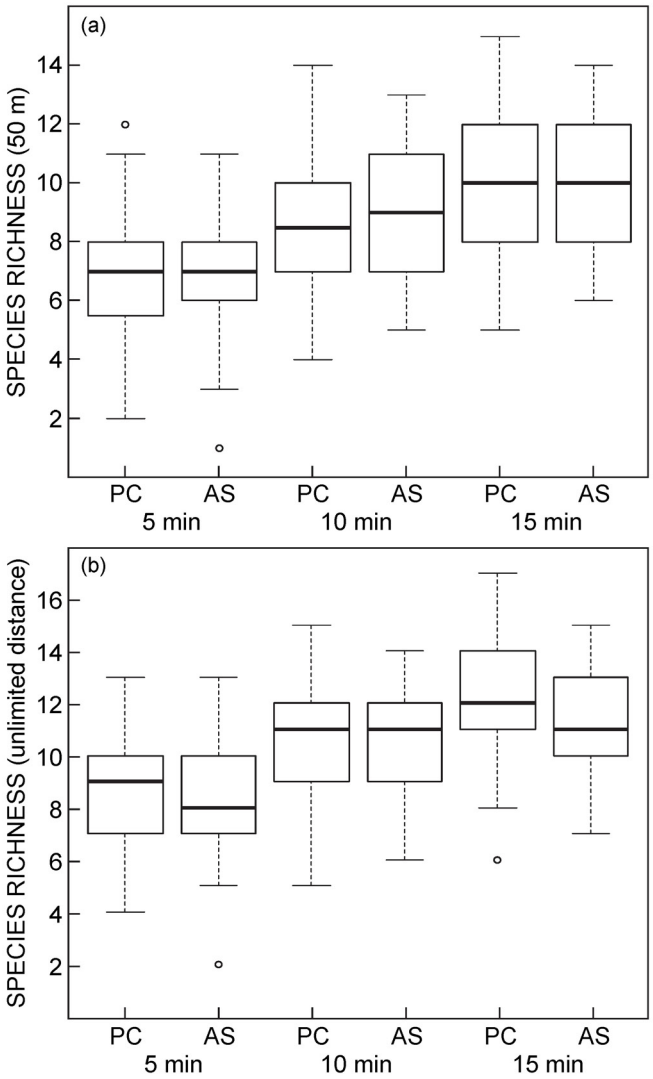


Figure 1: Cumulative species richness recorded at particular point samples (n = 48) using point counting in the field (PC) and listening to acoustic samples (AS) during 5-, 10- and 15-minute intervals within a radius of (a) 50 m from the observer/recorder (clearly audible individuals in acoustic samples) and (b) any distance from the observer/recorder. The boxplots show the median, interquartile range (box), and the minimum and maximum values (whiskers)

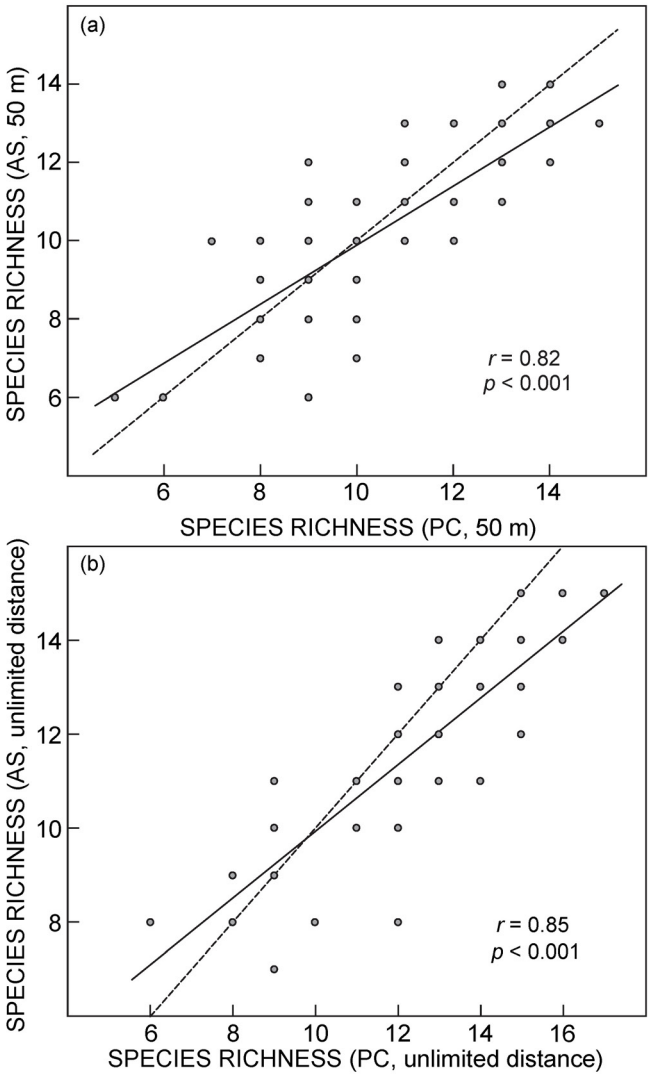


Figure 2: Correlation (solid line = trend in the data) of species richness recorded at particular point samples (n = 48) during 15 min. using point counting (PC) in the field and listening of acoustic samples (AS) within a radius of a) 50 m from observer/recorder (clearly audible individuals in acoustic samples) and b) any distance from observer/recorder. Dashed line shows the trend with the slope 1.0

$t = -3.89$, $p < 0.001$), but not after only 5 min (paired t-test, df = 47; 5 min: $t = -0.85$, $p = 0.40$). The species richness detected for particular 15-minute samples by the two methods was significantly correlated ($r = 0.85$, $p < 0.001$; Figure 2b). However, the number of cases below (29) the direct correlation (slope = 1) were comparably higher than those above the line (9) showing that for unlimited distance, acoustic sampling tends to overlook some species vocalising at distances greater than 50 m.

Species with a lower detectability during acoustic sampling compared to point counts were the Mountain Robin-Chat *Cossypha isabellae*, Cameroon Sunbird *Cyanomitra oritis*, Elliot's Woodpecker *Dendropicos elliotii* and African Thrush *Turdus pelios* (see Table 1 for raw data). On the other hand, species with more detections during acoustic sampling were Chubb's Cisticola *Cisticola chubbi*, Brown-throated Wattle-eye *Platysteira cyanea* (both only up to 50 m), Oriole Finch *Linurgus olivaceus*, Dusky Flycatcher *Muscicapa adusta* and Mount Cameroon Speirops *Speirops melanocephalus* (see Table 1 for raw data).

Bird community similarity (Sørensen index) among particular sampling sites was significantly correlated for estimations using point counts and listening to acoustic samples for both 50 m radius ($r = 0.79$, $p < 0.001$; Figure 3a) and unlimited distance ($r = 0.69$, $p < 0.001$; Figure 3b). However, the bird community similarity among individual census points tends to be underestimated if calculated from data based on listening to acoustic samples. This discrepancy appears to be more pronounced for the unlimited distance data set (Figure 3a and b).

We also calculated total abundances of particular species for the whole study site as a sum of maximum abundances estimated for 16 census points (Table 1). We observed a strong correlation between species abundances estimated by the two methods for both 50 m radius ($r = 0.92$, $p < 0.001$; Figure 4a) and unlimited distance ($r = 0.93$, $p < 0.001$; Figure 4b). However, the number of points above the direct correlation line (slope = 1.0) was lower for both distances (50 m: 10 above, 12 below; unlimited distance: 9 above, 16 below), showing that acoustic sampling underestimates overall abundances of several bird species (Figure 4a and b). Species whose abundances have been typically underestimated by acoustic sampling were mainly species occurring in flocks (Waller's Starling *Onychognathus walleri* and Western Mountain Greenbul *Andropadus tephrolaemus*), most abundant species with more individuals singing at a census point (Northern Double-collared Sunbird *Cinnyris reichenowi*) including duet species (Yellow-breasted Boubou *Laniarius atroflavus* and Grey Apalis *Apalis cinerea*), and a species that was identified only by warning calls (African Thrush *Turdus pelios*).

Discussion

The use of acoustic signals for detecting birds is crucial for surveys focused on estimating community composition, species richness and abundances of particular species (Dawson and Efford 2009). This holds true especially in densely vegetated habitats, including tropical woodlands and rain forests, where most individuals are more easily detected by sound than by sight (Celis-Murillo et al. 2012). During our field surveys, we recorded c. 93% of all individuals acoustically and more than half of all species was detected exclusively by voice. All species (with one exception, the uncommon Black-billed Weaver *Ploceus melanogaster*) that were detected during point counts were also recorded by listening to acoustic samples. This suggests that employing soundscape recordings is a reliable approach to produce complete checklists of bird communities, especially at places with a high rate of singing activity of most of the community members (Celis-Murillo et al. 2012).

Our study design was, however, focused mainly on estimating species richness and community composition of particular census points and evaluating the spatial limits of the method based on listening to acoustic samples. Estimates of local species richness were similar for point

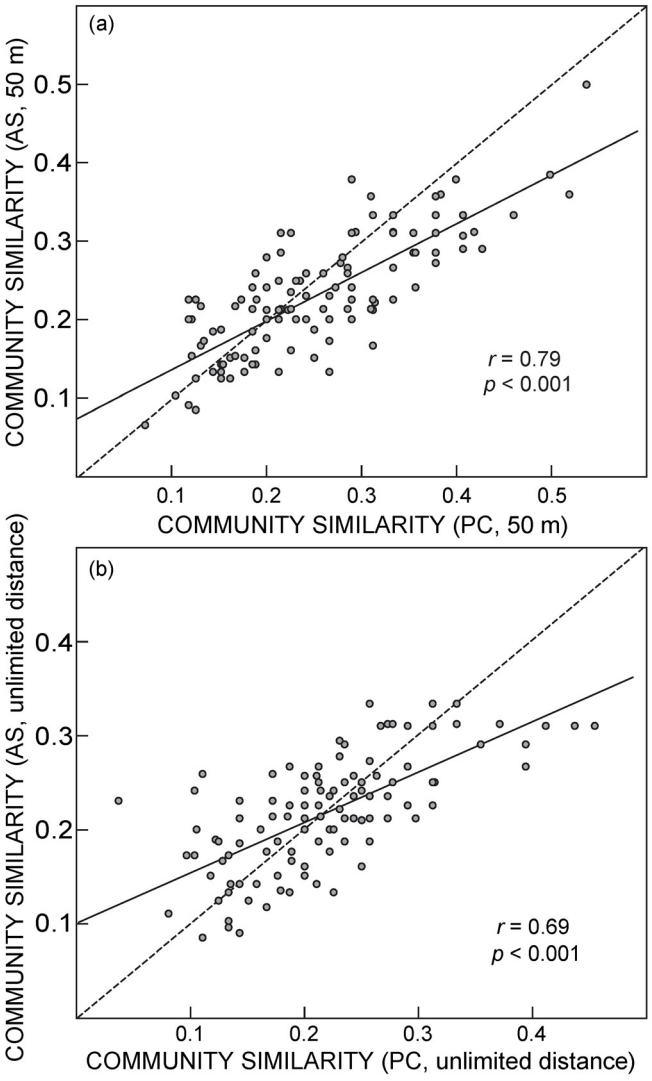


Figure 3: Correlation of bird community similarity (Sørensen index) among particular point samples ($n = 48$) estimated by point counting (PC) in the field and listening to acoustic samples (AS) within a radius of (a) 50 m from the observer/recorder (clearly audible individuals in acoustic samples) and (b) any distance from the observer/recorder. The dashed line shows the trend with the slope 1.0

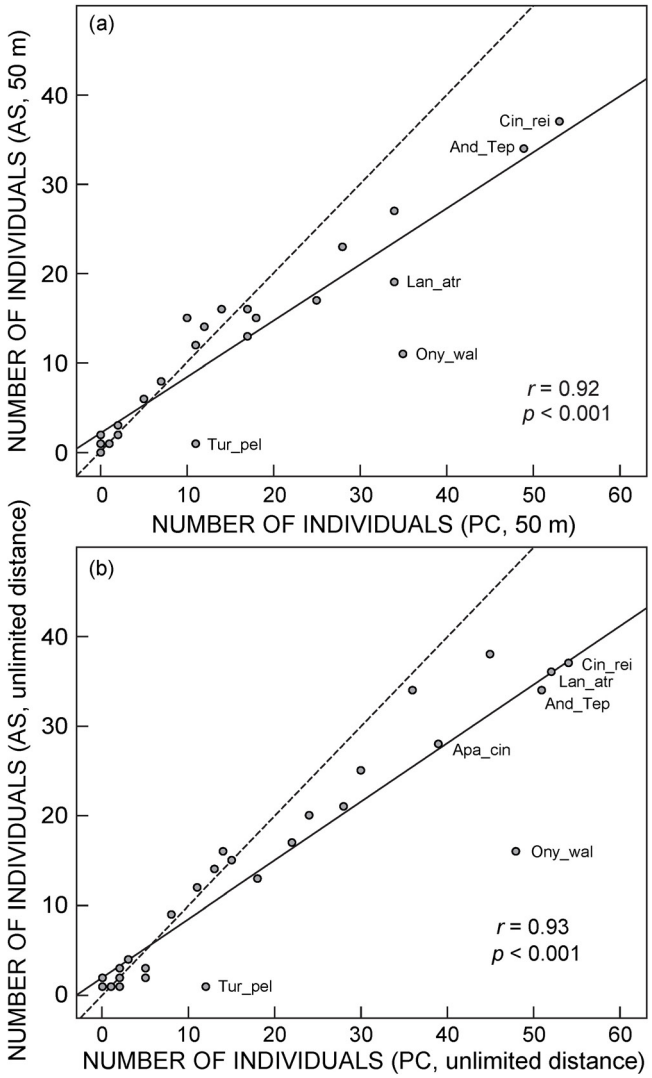


Figure 4: Correlation of total abundances of particular bird species estimated for 16 census points using point counting (PC) in the field and listening to acoustic samples (AS) within a radius of (a) 50 m from the observer/recorder (clearly audible individuals in acoustic samples) and (b) any distance from the observer/recorder. The dashed line shows the trend with the slope 1.0. Abbreviations correspond to the first three letters of the genus and species epithets listed in Table 1

count surveys and listening to acoustic samples in our study. This holds true especially for the distance up to 50 m, total species richness estimates for the 15-minute period, as well as cumulative estimates for all three 5-minute periods. Still, both methods provided similar diversity estimates for any distance. However, listening to acoustic samples tended to miss some individuals singing at greater distances. We observed such an underestimation in cumulative species richness estimates for 10- and 15-minute periods. It might be explained by the fact that the signal-to-noise ratio significantly decreases the detectability of some bird species beyond the 50 m distance (Rempel et al. 2013). Our previous studies that focused on investigation of bird community structures in the montane forests of the Cameroon volcanic line (Reif et al. 2006, 2007; Hořák et al. 2010; Djomo et al. 2014) showed that the narrow fixed radius of 50 m represents a reasonable compromise in montane rain forest as the detectability of all bird species seems to be comparable for this distance. On the other hand, a radius covering a much smaller area (e.g. 25 m) might not provide sufficient data on species richness and abundances.

An important question is how to estimate an approximate radius of 50 m during listening to acoustic samples. This undoubtedly requires a good knowledge of the singing activity of all species of a given community, especially with regard to species-specific song volume and its transfer efficiency in a given environment (Catchpole and Slater 2008). However, our experience of listening to SongMeter acoustic samples showed that most of the bird species are clearly audible for closer distances of approximately 50 m and individuals singing at a greater distance are well distinguishable. Two groups of birds seem to deviate from this rule in our study. (1) Bird species with loud vocalisations, e.g. *Platysteira cyanea*, and species that duet, namely *Laniarius atroflavus* or *Cisticola chubbi*. Our analysis showed that there is a tendency to include these species more frequently at closer distance (clearly audible) during listening to acoustic samples compared with reality. For this reason, these species can sometimes produce false positive records for closer distances and may contribute to slight overestimation of species richness. (2) Group of birds with relatively quiet singing. Surprisingly, according to our analysis this latter group of species can be relatively easily missed both during point counts (e.g. Oriole Finch *Linurgus olivaceus* and Dusky Flycatcher *Muscicapa adusta*) and listening to acoustic samples (e.g. *Cossypha isabellae*). Acoustic samples were also less efficient in detection of non-breeding species that vocalised only by short warning calls during our survey period (e.g. *Cyanomitra oritis* and *Turdus pelios*), and also less detectable was the tapping of woodpeckers (*Dendropicos ellioti* in the present case). It has been previously suggested that species with inconspicuous singing are more often missed by observers in the field when several species are singing and calling at the same time (Hutto and Stutzman 2009). Celis-Murillo et al. (2009) also suggested that the first several minutes of a bird survey might be challenging for observers because of the large number of new detections. They also noted that an advantage of acoustic samples is that sounds can be replayed to allow detection of individuals that might otherwise have been overlooked. Our results only partly conform with these studies, because we experienced that some individuals of species with inconspicious singing may be missed also during listening to acoustic samples.

Listening to acoustic samples also produced similar bird species checklists for particular census points if compared with data obtained by point count surveys. Therefore, we suggest that recordings can be used for an accurate estimation of bird community composition at local scales. We also showed that data based on listening to acoustic samples produce reliable estimates of beta diversity among particular census points. However, beta diversity tends to be slightly higher when estimated by listening to acoustic samples, especially for the sampling at any distance. This is likely caused by false negative detections for further distances.

Our study is the first to employ acoustic sampling for estimation of avian community structure in tropical Africa. Moreover, we are among the first showing that acoustic sampling provides a good estimate of species abundances for the purpose of interspecific comparisons. However, listening to acoustic samples rather failed in detecting more conspecifics singing at one particular census point (see also Celis-Murillo et al. 2009) and therefore typically led to underestimation of the total abundances of the most common species. In our study area, this was true mainly for species moving in flocks (*Onychognathus walleri* and *Andropadus tephrolaemus*), species with lekking singing behaviour (*Cinnyris reichenowi*) and abundant duetting species (*Laniarius atroflavus* and *Apalis cinerea*). Number of individuals was undoubtedly underestimated also for non-breeding species that had no or low singing activity (e.g. *Turdus pelios* and *Cyanomitra oritis*).

We conclude that listening to acoustic samples offers an alternative and useful approach for surveying bird communities that might accelerate the collection of basic information on community structures in poorly known Afrotropical regions and habitats. In addition, it is helpful when there is a need to monitor species-rich communities throughout the year (Sueur et al. 2012). Furthermore, the method produces archival records of surveys, providing an opportunity for detailed reanalyses of recordings such as automatic detections of particular species (e.g. Kasten et al. 2010; Goyette et al. 2011) and estimates of acoustic species richness (Depraetere et al. 2012; Gasc et al. 2013). These are gradually developing fields of research, which will present opportunities for a variety of studies much needed in the afrotropical region.

Acknowledgements — We wish to thank the local community of Bokwango village for their kind support, and especially to Francis, Nathaniel and Collins for their guarding during our fieldwork. We thank the Department of Environmental Sciences of the University of Buea, Cameroon and in particular Eric Fokam, Philip Tem Dia and Bertilla Sirri for their kind assistance. We also thank Kevin Njabo and the IRTC of the UCLA in Yaoundé for their technical support. This study was performed with the authorisation number 2309/PRBS/MINFOF/SG/DFAP/SDVEF/SC of the Ministry of Forestry and Wildlife of the Republic of Cameroon and with the support of Mt Cameroon National Park authorities. The research was funded by the Czech Science Foundation (project no. P505/11/1617) and UNCE.

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Received 30 June 2013, revised 10 February 2014, accepted 2 October 2014
Associate Editor: Stuart Taylor

Chapter 3

Vokurková J, Motombi F, Ferenc M, Hořák D, Sedláček O (2018). Seasonality of vocal activity of a bird community in an Afrotropical lowland rain forest. *Journal of Tropical Ecology*, 34, 53–64.

Journal of Tropical Ecology (2018) **34**:53–64. © Cambridge University Press 2018
doi:10.1017/S0266467418000056

Seasonality of vocal activity of a bird community in an Afrotropical lowland rain forest

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(*Received 21 January 2017; revised 2 February 2018; accepted 2 February 2018*)

Abstract: Recent observations from the tropics indicate seasonal peaks in breeding and vocal activity of some bird species. However, information about seasonality in vocal activity at the community level is still lacking in the tropics. We examined seasonal variation in the diurnal vocal activity of lowland rain forest birds on Mount Cameroon, using weekly automatic sound recording throughout the whole year and related it to rainfall and temperature. We show that the bird community in lowland rain forest vocalized year-round, but species richness as well as the vocal activity of the community varied greatly during the year. This variation coincided with the seasonality of rainfall. The highest number of species (31.5 on average) sang at the beginning of the driest period, followed by a gradual decrease in singing with increasing rainfall (minimum 14.5 species). This indicates that intensive rainfall indirectly limits the vocal activity of the tropical rain-forest bird community. Temporal turnover of vocalizing species as well as within-day variation in vocal activity was highest during the transition period between dry and rainy seasons. We suggest that this could reflect differing timing in the breeding activity of particular feeding guilds to follow seasonal peaks of their diets.

Key Words: Afrotropics, birds, lowland rain forest, seasonality, song

INTRODUCTION

The exact timing of reproduction is a critical process, which also determines the reproductive success of individuals (McNamara *et al.* 2011). In birds, breeding in extratropical zones is restricted to the spring and early summer, and the main proximate factor that controls the exact timing is the change in day length (Slater & Mann 2004, Wikelski *et al.* 2000). The seasonality of avian reproduction is generally less clearly defined in the tropics (Ricklefs 1969). Close to the equator, there is little seasonal change in temperature and day length (Dawson *et al.* 2001, Moore *et al.* 2005) but most tropical regions show marked seasonality in precipitation (Peres 1994, but see Tallman & Tallman 1997). Rainfall variability is considered a key factor in determining overall primary productivity (Ahumada 2001, Schloss *et al.* 1999), the phenology and fruit production of plants (van Schaik *et al.* 1993) and the abundance and activity of insects (Osborne 2000), all of which have great influence on food availability to birds (Blake & Loiselle 1991, Poulin *et al.* 1992, Wolda 1980).

The few available multi-species studies on indirect indicators of breeding, such as the presence of brood patches or moulting, have shown that the response of tropical bird communities to the variation in rainfall and food availability can be very complex and variable among geographic regions, biomes and functional groups of birds (Cox *et al.* 2013, Johnson *et al.* 2012, Serle 1981, Stouffer *et al.* 2013). Most of the bird species in the African savannas (Cox *et al.* 2013) and the central Amazonian and Panama rain forests (Brawn *et al.* 2017, Hau *et al.* 2008, Johnson *et al.* 2012, Loiselle 1988) breed at any time of the year even though bird communities show distinct seasonal peaks in the number of species and individuals breeding (Williams & Middleton 2008). However, data on the breeding activity of birds over the course of the year are still scarce from many tropical regions and biomes with variable rainfall patterns.

In the tropics, with relatively little seasonal environmental change, a high proportion of bird species is resident and a significant proportion of them defends territories year-round (Stutchbury & Morton 2008). Under these conditions, it is generally expected that singing

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activity is relatively constant year-round and that song is also used outside of the breeding context for territory defence (Tobias *et al.* 2011, Topp & Mennill 2008). However, recent studies on several Neotropical species have indicated that singing activity varies throughout the year – males sing infrequently outside of the breeding season and increase their singing activity dramatically during the breeding period (Chiver *et al.* 2015, Hill *et al.* 2015, Koloff & Mennill 2013). Song, often performed by both sexes, also functions in stimulation of the social mate and helps in within-pair timing and the synchronization of nesting in the tropics (Chiver *et al.* 2015, Kunkel 1974). There is an increasing evidence that to some extent singing activity also reflects breeding activity in tropical bird communities (Catchpole & Komdeur 1993, Chiver *et al.* 2015, Hill *et al.* 2015, Moore *et al.* 2004). However, there is a fundamental lack of studies examining community-level year-round vocal activity from tropical regions.

In this study, we attempted to fill this gap by examining seasonal variation in the vocal activity of a bird community in an Afrotropical lowland rain forest in the foothills of Mount Cameroon, using a newly developed automatic sound recording approach. Specifically, we address the following hypotheses: (1) The species richness of the vocalizing bird community and (2) the vocal activity of the bird community will vary seasonally in relation to seasonal changes in temperature and precipitation. (3) The temporal turnover in the composition of the vocalizing bird community will be highest during the transition periods between the dry and rainy season.

METHODS

Study site

The study was conducted in the interior of a primary lowland rain forest in the Mt Cameroon National Park. Mt Cameroon (4095 m asl) rises from the Atlantic coast in the Gulf of Guinea in the south-west region of Cameroon, and is part of an extensive volcanic chain extending from the coast to about 1000 km inland in a north-east direction (DeLancey & DeLancey 2000). Soundscape recordings were taken at the foothills of the south-west slope of Mt Cameroon, about 3 km north-west of the seashore and Bakingili village (near Bamboo Camp site; 4°06′46″ N, 9°04′06″ E) at an altitude of 350 m asl. The pristine part of the lowland forest starts from about 300 m asl; forests below this altitude have been replaced by plantations and secondary growth. Lowland forest at the study site has a closed canopy (almost 100%) and sparse undergrowth. In total, 124 tree species (>10 cm dbh) were identified at 16 sampling plots (~2 ha area in total) at the study altitude. Maximum and mean tree heights were 48 and 16 m, respectively (Djomo Nana *et al.* 2015).

Environmental variables

The mean annual precipitation at our study site is ~7000 mm (Gonfiantini *et al.* 2001). For our analyses, we used meteorological data from the nearest Mokundange meteorological station (Fraser *et al.* 1998), which is located about 13 km south-east of our study site (4°1′3.64″ N, 9°8′24.71″ E, 50 m asl). The annual precipitation is lower there (mean 5000 mm) compared with our site, but the relative distribution of precipitation over the course of the year is very similar (Cable & Cheek 1998, see Fraser *et al.* 1998 for details). The data for temperature are from 1971–1975 and 1984–1993, and data for precipitation are from 1970–1993. We calculated monthly averages from these periods for minimum and maximum temperature and amount of rainfall and number of rainy days. To our knowledge, no other detailed and/or more recent long-term meteorological data for the Mt Cameroon region are available. We collected temperature and precipitation data at the study site for 1 y (2013 for temperature and 2015 for precipitation), which, however, do not correspond to the year of our acoustic recording. We show in Appendix 1 that the environmental data from a single recent year show almost identical patterns to the long-term data, which we used for the analyses. The rains in this region occur year-round, but dry season can be defined as the period between November and April. The winds blowing southward from the Sahara have the strongest influence on climate during this period. The dry season is broken by south-western winds in late April or May causing a steep increase of rainfall. By June or July, the rain falls almost continuously but is less intense. The peak of rainy season is in August with quite continuous decrease during September and October (Fraser *et al.* 1998 and Figure 1).

Acoustic sampling

We sampled the vocal activity of the bird community from 2 December 2011 until 12 November 2012. We used Song Meters (SM2, Wildlife Acoustics, Inc., Concord, MA, USA) for soundscape acoustic sampling with automatic recording devices and two omnidirectional microphones supplied with SM2 (SMX-II Weatherproof Acoustic Microphone; for detailed description of Song Meter recording devices see Sueur *et al.* 2012). The devices were mounted on a tree stem in the forest interior at a height of ~180 cm and placed ~200 m apart to avoid overlapping acoustic sampling. The sampling frequency was 44.1 kHz, and 16 bit recordings were

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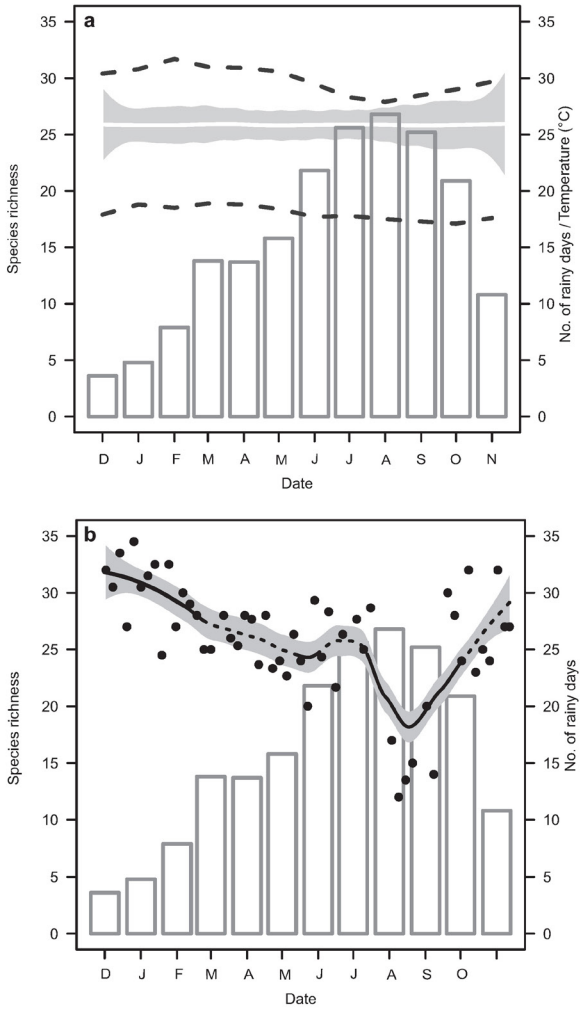


Figure 1. The expected seasonal patterns of species richness under the null model (white line – mean, shaded areas – 95 % confidence interval). Bars depict monthly number of rainy days; dashed lines represent mean minimum and maximum temperatures in Mt Cameroon area (a). Seasonal trend in the real species richness (the number of bird species detected per day) between 2 December 2011 and 12 November 2012 in the lowland rain forest of Mt Cameroon. Dots represent means from available recording devices, lines are trends in the data fitted by locally weighted regression (LOESS) function, with shaded areas representing standard error estimates (b). The significant deviation from the null model is indicated by solid line. Bars depict monthly number of rainy days.

saved in WAV format on 32GB SDHC cards. The batteries and cards were changed after 5 mo. The devices were programmed to record every sixth day continuously for 3 h in the morning, starting 20 min before sunrise (the device automatically calculates the actual sunrise time according to the GPS position). This study design was chosen so as to cover the daytime with the highest vocal activity of birds while taking into account the battery lifetime. In total, we placed four devices at the sampling site. However, only one of them worked for the whole year. Even though the devices are advertised as being waterproof (including the microphones), three of the four recording devices stopped working during the wet period of the year due to extremely high rainfall and humidity at the study site. In total, we obtained recordings from 59 d, which corresponds to 129 device-days (387 h). We checked all samples by listening and omitted all recording days with rain. This resulted in 53 d and 116 device-days, which corresponds to 812 5-min samples in total used for further analyses (~68 h). The coverage of the year by individual devices is shown in Appendix 2.

Data analysis

To estimate the vocal activity of the bird community, we selected seven 5-min samples from each 3-h recording resulting in 35 min per sampled day in total. Altogether, we analysed 4060 min of data from four devices over the year. These samples were cut manually using GoldWave 5.67 software (GoldWave Inc., St. John's, NL Canada), using even spacing every 30 min throughout the length of the recording. We applied this subsampling to reduce the length of recordings to listen to, while keeping representative time frames for evaluating the bird vocal activity at the same time. Samples were checked by FNM, an experienced local expert, who listened to the original WAV files using a computer (played in GoldWave) and stereo headphones under standardized conditions. Each recording was played with a volume approximately simulating natural conditions. Each individual that was heard singing was noted to compile a list of species recorded during each 5-min acoustic sample. Our analysis is based on typical songs, calls or other vocalizations of all birds, including non-passerines. Our previous analyses have shown that listening to acoustic samples produces reliable data on species richness for an approximately 50-m radius around the recording device (Song Meter) in the tropical rain-forest (Sedláček *et al.* 2015).

First, we asked how many species vocalized per day. We quantified the number of bird species (hereafter species richness) recorded during all seven samples for a particular day and device (recorded during 35-min in total for each device), and this species richness was averaged across the available devices for a given recording day. We constructed a null model by randomizing the vocal activity of individual bird species within the year to quantify the expected species richness patterns if the species were vocally active irrespective of the season. Subsequently, we quantified the 95% confidence interval of species richness that would be observed under such a scenario (Figure 1a). Real species richness values that fall outside of this confidence interval were considered as significant deviations from the null model. Second, we

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estimated the overall vocal activity of the bird community (hereafter mean vocal activity of species) in particular days. We quantified this parameter as the average number of 5-min intervals in which particular species vocalized (i.e. the number of presences in seven 5-min samples averaged across species for a particular day) with subsequent averaging across available devices. This measure reaches its highest values in cases where many species are vocalizing during all seven 5-min intervals during a particular morning. Further, we estimated the daily variance (computed as statistical variance) in the number of species detected across the seven 5-min samples to quantify the variability of vocal activity (hereafter variance in richness of vocalizing community) within a given day. We averaged this variance across available devices. This measure quantifies if the vocal activity of a bird community is constant or varies among samples during the morning. We plotted these variables against the recording Julian date and utilized the locally weighted regression (LOESS) function. To explore the dependence of species richness of the vocalizing bird community on climatic variables, we calculated the average number of bird species recorded by the devices in each month (as only monthly climatic data were available). Monthly species richness was thereafter regressed on minimum and maximum monthly temperature (averaged across the years 1971–1975 and 1984–1993), amount of rainfall and the number of rainy days in each month (averaged across the years 1970–1993).

Changes in composition of the vocally active avian community throughout the year were quantified by two beta-diversity indices β_{SIM} (species replacement) and β_{NES} (nestedness) introduced by Baselga (2010). These indices reflect two different phenomena, which can produce differences in species composition between two sample units. The first phenomenon is the actual replacement of some species by others. The second phenomenon is nestedness, purely reflecting differences in species richness in the compared samples. In the analysis of temporal beta-diversity, we used only data from the single recording device that operated year-round. We calculated beta-diversity indices by comparing consecutive recording days. The LOESS function was utilized to visualize these temporal trends. All analyses were conducted in R software 3.5.4.

RESULTS

Seasonal variation in species richness and vocal activity

In total, we detected 102 bird species during 53 recording days (all detected species and their frequency of occurrence listed in Appendix 3). The mean number of species detected per day varied between 14.5 species (in August) and 31.5 species (in December). The species richness showed a clear trend with the highest species richness at the beginning of the dry season between December–January (higher than null model expectations, Figure 1a, b), followed by gradual decrease with increasing rainfall towards June (lower than null model expectations, Figure 1a, b). A slight increase of species richness by June/July just before the beginning of the heaviest rains was followed by a steep decline during July, and low species richness during the peak of the rainy season in August–September (lower than null model expectations, Figure 1a, b). Species richness of vocalizing birds then steeply increased with the rainfall decline during October–November (Figure 1b).

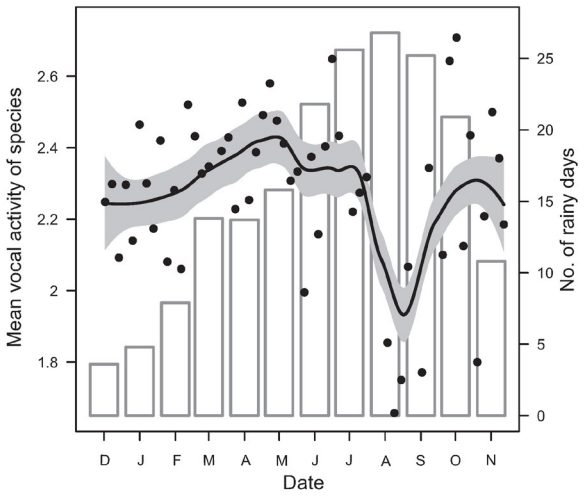


Figure 2. Mean vocal activity of species (average number of 5-min intervals in which each species was detected) between 2 December 2011 and 12 November 2012 in the lowland rainforest of Mt Cameroon. Dots represent means from available recording devices, solid lines are trends in the data fitted by locally weighted regression (LOESS) function, with shaded areas representing standard error estimates. Bars depict monthly number of rainy days.

The mean vocal activity of species had a similar trend but rather increased towards the end of dry season (from December to the end of April), then again steeply declined during the peak of the rains (July–August), and increased with decreasing rainfall by the end of September and in October (Figure 2). The mean number of species detected per 5-min sample within a day varied between 3.7 species (August) and 10.1 species (December), and also corresponded to the rainfall patterns throughout the year.

Variance in richness of vocalizing community

The statistical variance in the number of vocalizing species detected in the seven 5-min samples on particular

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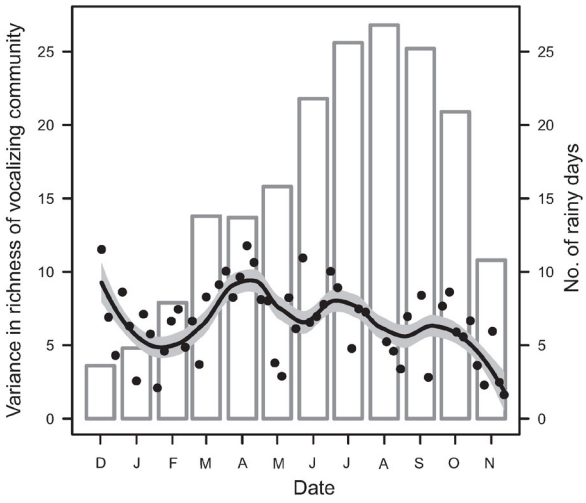


Figure 3. Seasonal trend of the variance in richness of vocalizing community (variance in the number of species vocalizing per 5-min samples within a particular day). Dots represent means of variance across available recording devices. Solid line shows the trend in the data fitted by locally weighted regression (LOESS) function with the shaded area representing standard error estimate.

days (averaged for recording units) was highest between late March and June (the transition period between the dry and rainy seasons) and then gradually decreased towards November–December (beginning of the dry season) (Figure 3). This result together with Figure 2 indicates that the bird community vocal activity throughout the morning is relatively stable and high during the dry season, highly variable during the transition period between the dry and rainy seasons (within a day, 5-min samples with both relatively low and high activity were detected) and stable and low during the peak of rains.

The influence of environmental seasonality on species richness

The species richness of the vocalizing bird community was related to seasonal changes in temperature and rainfall (these environmental variables are strongly negatively correlated, Appendix 4). We found that the species richness of birds detected vocalizing during individual months was negatively related to the number of rainy days per month ($R^2 = 0.57$, $n = 12$, $P < 0.01$; Figure 4a) as well as to the monthly amount of rainfall ($R^2 = 0.60$, $n = 12$, $P < 0.01$; Figure 4b), and positively related to the monthly mean maximum temperature ($R^2 = 0.44$, $n = 12$, $P < 0.05$; Figure 4d). The relationship was positive but not significant for monthly mean minimum temperature ($R^2 = 0.20$, $n = 12$, $P = 0.15$; Figure 4c). The species richness of birds vocalizing during the dry season (months with less than 5 rainy days and higher temperature) was more than twice as high as during the peak of the rainy season (months with more than 25 rainy days and lower temperature) (Figure 4a, b).

Seasonal changes in the composition of the vocalizing bird community

The composition of the vocalizing bird community changed throughout the year. Temporal species turnover (beta sim β_{SIM}, Figure 5) of the singing bird community was highest during the transition period between the dry and rainy seasons between March and June (with a peak in April–May), and relatively low during heavy rains (July–August) and the dry season (December–January). On the other hand, the nestedness component of beta diversity (β_{NES}, Figure 5) attributable to temporal changes in species richness showed the opposite pattern. The vocalizing bird community was more nested during the peak of rains (July–September) than during the transition period between the dry and rainy seasons (March–June).

DISCUSSION

To our knowledge, although based on relatively limited sample size, this is the first study to quantify seasonal patterns in the vocal activity of birds in the tropics at the community level. We show that birds in this lowland rain forest vocalized year-round, but that the number of singing species varied greatly throughout the year and this variation coincided mainly with seasonality of the rainfall. The species composition of the vocalizing community changed mainly during the transition period between the dry and rainy seasons, and was relatively stable during both heavy rains and the driest months. In the periods of the highest community change there was also the highest variation (i.e. variance in richness of vocalizing community), meaning that there were short intervals of very high and also low vocal activity within a single day.

We show that the singing performance of the bird community at our study site varied greatly depending on the season. The two measures of singing performance show similar patterns – during periods of high species richness, the overall vocal activity of birds (mean vocal activity of species) was also high. Our analyses also show that the amount of rainfall was the most important factor negatively influencing the vocal activity of rain-forest birds. It is important to highlight here that we omitted acoustic samples taken during rain from our analyses and related the species richness to the rainfall in particular months. This means that the singing behaviour of birds

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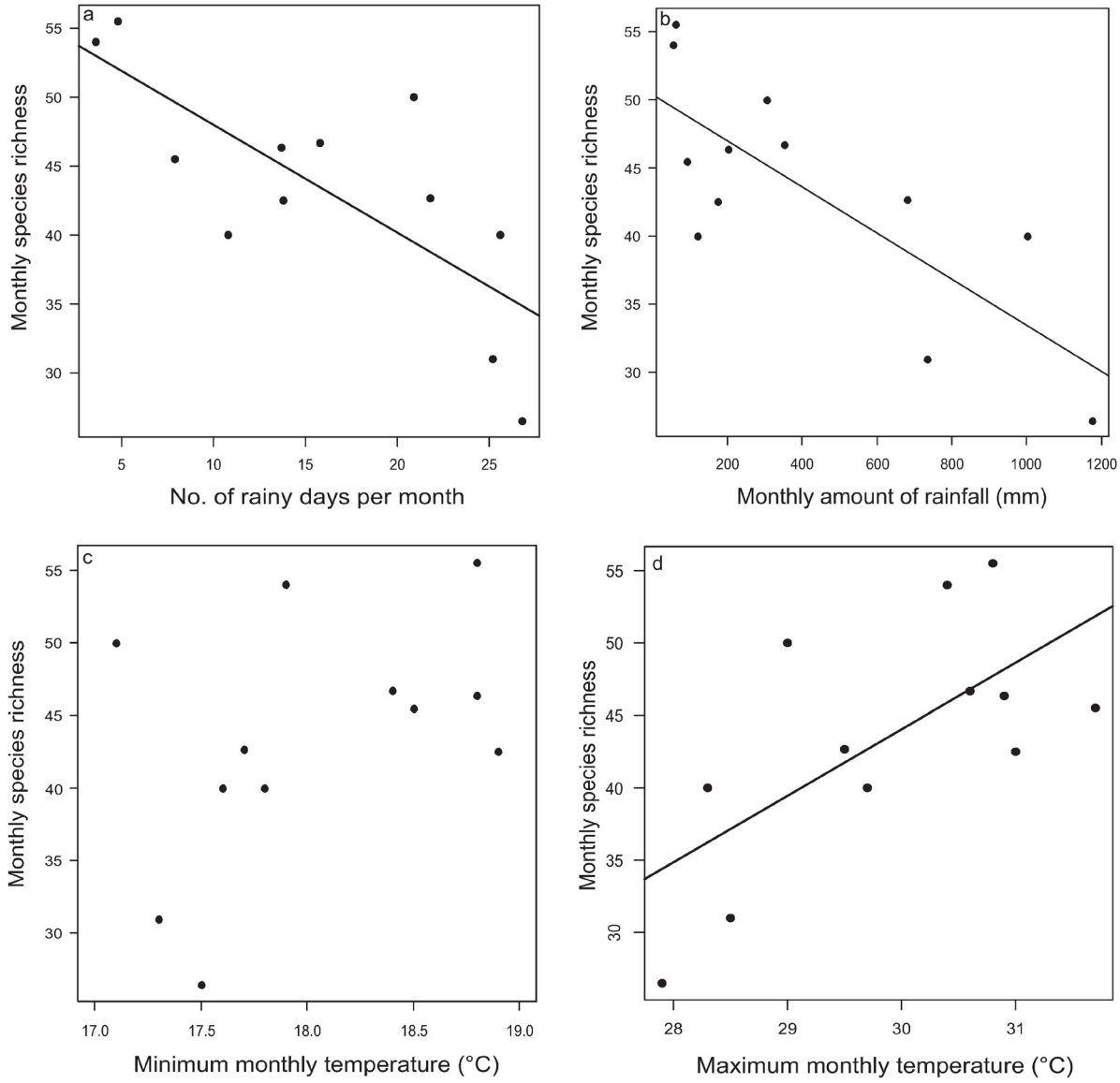


Figure 4. Relationships between the monthly species richness of birds vocalizing in particular months, and the number of rainy days per month ($R^2 = 0.57$, n = 12, $P < 0.01$) (a), monthly amount of rainfall ($R^2 = 0.60$, n = 12, $P < 0.01$) (b), mean monthly minimum temperature ($R^2 = 0.20$, n = 12, $P = 0.15$) (c) and mean monthly maximum temperature ($R^2 = 0.44$, n = 12, $P < 0.05$) (d) in the lowland rain forest of Mt Cameroon. Dots represent means from available recording devices. The depicted trend was fitted by the ordinary least squares method.

was therefore reduced not only during particular rain events, but more interestingly also during the wet season in general. We propose that the main mechanism explaining these findings is not the rainfall per se, but rather its effect on the availability of organisms (plants, insect, vertebrates) that are part of the feeding source of the birds. Strong rainfall and decreased temperature during the peak of rains significantly reduce the availability of invertebrate prey for birds. The invertebrate prey in the foliage and especially aerial forms of insects are less active and therefore hidden for birds during periods of heavy rains (Foster 1974, Pearson 1977, Senapathi *et al.* 2011). Further, despite that fact that we do not have information on the phenology of plants and trees for our study region, it is possible that the availability of flowers and fruits is reduced during the peak of rains (Anderson *et al.* 2005, van Schaik *et al.* 1993). Singing, as a possible signal of territory quality (Manica *et al.*

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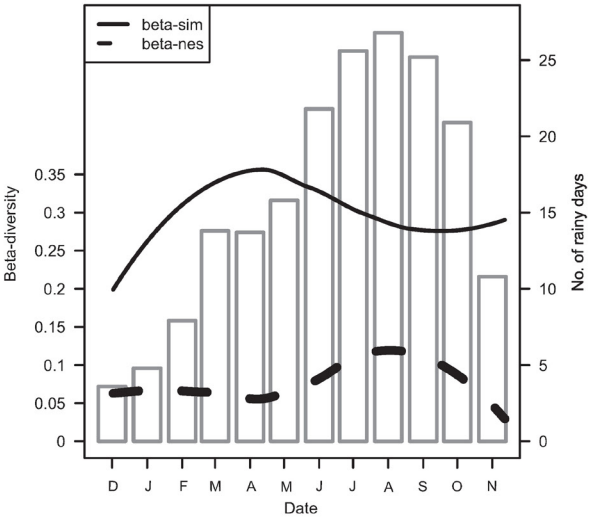


Figure 5. Temporal change in the composition of the vocalizing bird community throughout the year quantified by β_{SIM} values (temporal species turnover) and β_{NES} values (nestedness). Data are from the single recording device that operated year-round in the lowland rain forest of Mt Cameroon.

2014), may be therefore omitted or highly reduced during periods of food shortage, as birds focus mostly on foraging during relatively limited periods of time. Alternatively, another possible explanation of higher species richness during the dry season might also be enrichment of the lowland bird community by montane altitudinal migrants (Werema *et al.* 2016). However, we did not detect any montane species singing in our recordings, even though altitudinal migrations of montane birds to lowland have been described from Mt Cameroon (Stuart 1986).

Information on the connection of singing performance to breeding activity is still missing for the vast majority of species in the tropics. However, recent studies have proposed that males of tropical birds sing infrequently outside of the breeding season and increase their singing activity dramatically during the breeding period (Chiver *et al.* 2015, Hill *et al.* 2015, Koloff & Mennill 2013). Data on the breeding activity of many bird species from our region, especially from the rainy season, are still scarce (Serle 1981, Tye 1992); however, we suggest that under the extremely high rainfall on Mt Cameroon many birds can avoid breeding during the peak of rains (Fraser *et al.* 1998). Males can reduce their vocal performance to save energy during periods of high metabolic demands and worsened conditions for acoustic signal transmission due to abiotic noise (Lengagne & Slater 2002, Mathevon *et al.* 2008). We also found that the birds responded very quickly to the drop in rainfall at the end of the wet season, as the mean vocal activity of species and also the number of species singing rapidly increased and was highest during the start of the driest period of the year on Mt Cameroon, which suggests that this is the period when most species breed (Serle 1981). It is also possible that birds respond to subtle changes in day length; alternatively, hormonal and physiological states of birds that are vocalizing might be affected by sunlight (Goymann *et al.* 2012). However, the proximate and ultimate mechanisms affecting bird singing behaviour in the tropics needs further investigation.

Nevertheless, our analyses on the species composition of the vocalizing bird community throughout the year imply that not all bird species have their peak of vocal activity during the driest months. We found that temporal species turnover (beta diversity) of the bird community increased with upcoming light rains and peaked during the transition period between the dry and rainy seasons (April–May). Moreover, the daily variance in the number of species vocalizing increased with forthcoming rainfall. This means that variable weather conditions and the first heavy rains in particular influenced the vocal behaviour of birds during this period. These results suggest that species with peaks of singing during the drier season decrease their vocal activity with increasing amounts of precipitation, whereas other species assemblages that prefer more humid periods of the year for breeding increase their vocal activity with upcoming rains. This leads to high species turnover during transitional periods of environmental conditions, suggesting that factors such as food composition and/or its availability can change dramatically (Hau 2001). The nestedness component contributed to beta diversity mainly during the rainy season, when the species richness of vocally active birds decreased.

Our results therefore support a view that bird reproduction may be more seasonal in Afrotropical rain forests than was previously thought, and different species might prefer different times of the year for breeding (Moreau 1950, Serle 1981). As has been previously shown, the timing of singing and breeding activities of particular feeding guilds may follow seasonal peaks in their diets (Greenberg 1995, Karr 1976, Mulwa *et al.* 2013). However, this issue needs further investigation and should be a main focus of future studies. Our study also shows that the use of automatic recordings is an effective tool for investigation of seasonal changes in singing activity of birds at the community level, especially in remote and hard-to-access tropical areas.

ACKNOWLEDGEMENTS

We wish to thank the local communities of the Bokwango and Bakingili villages for their kind support, and especially to Francis Escobar Luma, Nathaniel and Collins for their guarding during our fieldwork. We

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thank Eric Djomo Nana and Štěpán Janeček for help in the field. We thank the Mt Cameroon National Park and the Department of Environmental Sciences of the University of Buea, Cameroon and in particular Eric Fokam, Philip Tem Dia and Bertilla Sirri for their kind assistance. We also thank Kevin Njabo and the Congo Basin Institute in Yaoundé for their technical support. David Hardekopf improved the English of the paper. This study was performed with the authorization number: 0053/PRBS/MINFOF/SG/DFAP/SDVEF/SC of the Ministry of Forestry and Wildlife of the Republic of Cameroon. The study was funded by the Grant Agency of Charles University (project no. GA UK 1282214) and the Czech Science Foundation (project no. 14–36098G).

Conflict of Interest

The authors declare that they have no conflict of interest.

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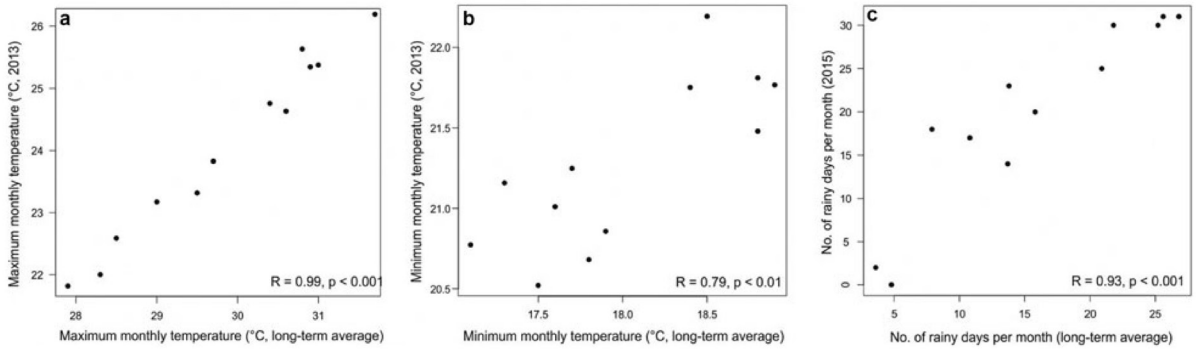
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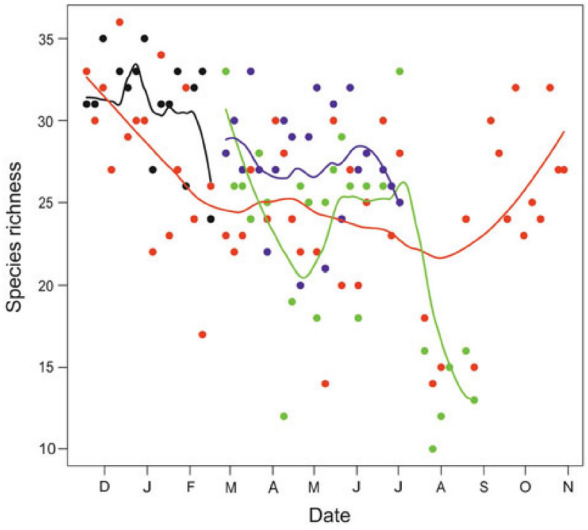
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APPENDICES



Appendix 1. The correlations of the environmental data from rain gauges and dataloggers obtained in the lowland rain forest (350 m asl) at the foothills of the south-west slope of Mt Cameroon during a single recent year (2015 and 2013, respectively) and of the long-term environmental data from the nearest Mokundange meteorological station (Fraser *et al.* 1998). The monthly average data are from 1971–1975 and 1984–1993 for maximum (a) and minimum (b) temperature, and from 1970–1993 for the number of rainy days per month (c).



Appendix 2. The coverage of the year by recordings from four individual devices (different colours) in the lowland rain forest (350 m asl) at the foothills of the south-west slope of Mt Cameroon. The dots show the number of species recorded per day by individual devices. The solid line shows the trend in the data fitted by locally weighted regression (LOESS) function. Days with rainfall are omitted from the analyses.

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Appendix 3. List of all detected bird species from recordings included into analyses of the vocal activity from 2 December 2011 until 12 November 2012 in the lowland rain forest (350 m asl) at the foothills of the south-west slope of Mt Cameroon. Sites – number of sites, where a species has been detected (min 1, max 4); Presence – number of days, when a species has been detected (min 1, max 53). The taxonomy follows Gill & Donsker (2017).

Species	Order	Family	Sites	Presence (d)
Accipiter tachiro	Accipitriformes	Accipitridae	3	14
Alethe diademata	Passeriformes	Muscicapidae	4	34
Anthreptes rectirostris	Passeriformes	Nectariniidae	2	2
Apalis nigriceps	Passeriformes	Cisticolidae	1	2
Apalis rufogularis	Passeriformes	Cisticolidae	2	4
Apaloderma aequatoriale	Trogoniformes	Trogonidae	2	6
Aquila africana	Accipitriformes	Accipitridae	1	1
Baeopogon clamans	Passeriformes	Pycnonotidae	4	7
Baeopogon indicator	Passeriformes	Pycnonotidae	4	18
Bathmocercus rufus	Passeriformes	Cisticolidae	1	1
Bleda notatus	Passeriformes	Pycnonotidae	4	25
Bleda syndactylus	Passeriformes	Pycnonotidae	4	22
Buccanodon duchaillui	Piciformes	Lybiidae	4	40
Bycanistes albotibialis	Bucerotiformes	Bucerotidae	0	2
Calyptocichla serinus	Passeriformes	Pycnonotidae	4	10
Camaroptera chloronota	Passeriformes	Cisticolidae	4	31
Camaroptera superciliaris	Passeriformes	Cisticolidae	0	1
Campethera nivosa	Piciformes	Picidae	0	2
Centropus leucogaster	Cuculiformes	Cuculidae	3	14
Ceratogymna atrata	Bucerotiformes	Bucerotidae	0	21
Ceratogymna elata	Bucerotiformes	Bucerotidae	4	15
Cercococcyx olivinus	Cuculiformes	Cuculidae	0	11
Ceuthmochares aereus	Cuculiformes	Cuculidae	3	12
Cinnyris superbus	Passeriformes	Nectariniidae	3	5
Circaetus spectabilis	Accipitriformes	Accipitridae	2	2
Clamator levaillantii	Cuculiformes	Cuculidae	1	2
Columba unicincta	Columbiformes	Columbidae	2	3
Corythaeola cristata	Musophagiformes	Musophagidae	4	28
Criniger calurus	Passeriformes	Pycnonotidae	4	41
Criniger calurus	Passeriformes	Pycnonotidae	4	36
Criniger ndussumensis	Passeriformes	Pycnonotidae	1	2
Cuculus clamosus	Passeriformes	Pycnonotidae	2	9
Cyanomitra cyanolaema	Passeriformes	Nectariniidae	4	31
Cyanomitra olivacea	Passeriformes	Nectariniidae	4	51
Deleornis fraseri	Passeriformes	Nectariniidae	4	49
Dicrurus atripennis	Passeriformes	Dicruridae	4	41
Eurillas ansorgei	Passeriformes	Pycnonotidae	4	34
Eurillas curvirostris	Passeriformes	Pycnonotidae	1	1
Eurillas gracilis	Passeriformes	Pycnonotidae	3	9
Eurillas latirostris	Passeriformes	Pycnonotidae	4	52
Eurillas virens	Passeriformes	Pycnonotidae	4	49
Eurystomus gularis	Coraciiformes	Coraciidae	2	2
Gymnobucco calvus	Piciformes	Lybiidae	2	5
Gymnobucco peli	Piciformes	Lybiidae	3	12
Gypohierax angolensis	Accipitriformes	Accipitridae	2	3
Halcyon badia	Coraciiformes	Alcedinidae	4	33
Halcyon malimbica	Coraciiformes	Alcedinidae	2	4
Hedydipna collaris	Passeriformes	Nectariniidae	4	33
Himantornis haematopus	Gruiformes	Rallidae	2	1
Hylia prasina	Passeriformes	Incertae Sedis	4	48
Chlorophoneus multicolor	Passeriformes	Macrosphenidae	1	1
Chloropicus xantholophus	Piciformes	Picidae	1	1
Chrysococcyx cupreus	Cuculiformes	Cuculidae	4	25
Chrysococcyx klaas	Cuculiformes	Cuculidae	3	4
Illadopsis cleaveri	Passeriformes	Pellorneidae	2	6
Illadopsis fulvescens	Passeriformes	Pellorneidae	3	16

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Appendix 3. Continued

Species	Order	Family	Sites	Presence (d)
Illadopsis rufipennis	Passeriformes	Pellorneidae	4	46
Ispidina picta	Coraciiformes	Alcedinidae	3	5
Ixonotus guttatus	Passeriformes	Pycnonotidae	3	6
Lamprotornis splendidus	Passeriformes	Sturnidae	2	2
Lophoceros fasciatus	Bucerotiformes	Bucerotidae	4	12
Macrosphenus concolor	Passeriformes	Macrosphenidae	4	40
Macrosphenus flavicans	Passeriformes	Macrosphenidae	4	45
Malimbus nitens	Passeriformes	Ploceidae	2	3
Melignomon eisentrauti	Piciformes	Indicatoridae	2	2
Muscicapa sethsmithi	Passeriformes	Muscicapidae	3	6
Neocossyphus poensis	Passeriformes	Turdidae	4	18
Nicator chloris	Passeriformes	Nicatoridae	2	34
Nigrita fusconotus	Passeriformes	Estrildidae	1	1
Nigrita luteifrons	Passeriformes	Estrildidae	4	12
Onychognathus fulgidus	Passeriformes	Sturnidae	2	6
Oriolus brachyrynchus	Passeriformes	Oriolidae	4	31
Oriolus nigripennis	Passeriformes	Oriolidae	4	8
Phyllastrephus albigularis	Passeriformes	Pycnonotidae	1	3
Phyllastrephus icterinus	Passeriformes	Pycnonotidae	4	36
Phyllastrephus xavieri	Passeriformes	Pycnonotidae	4	13
Platysteira castanea	Passeriformes	Platysteiridae	4	42
Platysteira concreta	Passeriformes	Platysteiridae	3	10
Platysteira tonsa	Passeriformes	Platysteiridae	4	31
Pogoniulus atroflavus	Piciformes	Lybiidae	4	35
Pogoniulus bilineatus	Piciformes	Lybiidae	1	1
Pogoniulus scolopaceus	Piciformes	Lybiidae	4	14
Pogoniulus subsulphureus	Piciformes	Lybiidae	4	48
Polyboroides typus	Accipitriformes	Accipitridae	1	1
Pseudalethe poliocephala	Passeriformes	Muscicapidae	4	19
Psittacus erithacus	Psittaciformes	Psittacidae	2	3
Sarothrura elegans	Gruiformes	Sarothruridae	1	2
Sarothrura pulchra	Gruiformes	Sarothruridae	2	7
Smithornis rufolateralis	Passeriformes	Eurylaimidae	3	18
Stelgidillas gracilirostris	Passeriformes	Pycnonotidae	3	7
Stiphrornis erythrothorax	Passeriformes	Muscicapidae	3	14
Stizorhina fraseri	Passeriformes	Turdidae	4	29
Sylvietta denti	Passeriformes	Macrosphenidae	4	6
Tauraco macrorhynchus	Musophagiformes	Musophagidae	4	52
Terpsiphone batesi	Passeriformes	Monarchidae	1	1
Terpsiphone rufiventer	Passeriformes	Monarchidae	4	47
Trachyphonus purpuratus	Piciformes	Lybiidae	3	17
Treron calvus	Columbiformes	Columbidae	4	29
Tricholaema hirsuta	Piciformes	Lybiidae	4	37
Trochocercus nitens	Passeriformes	Monarchidae	4	41
Turtur brehmeri	Columbiformes	Columbidae	4	42
Turtur tympanistria	Columbiformes	Columbidae	3	6

Appendix 4. Pearson's product-moment correlation coefficient (r) for environmental variables (number of rainy days per month, monthly rainfall, maximum and minimum monthly temperature) from long-term data (Fraser *et al.* 1998) included into analyses of the species richness of the vocalizing bird community from 2 December 2011 until 12 November 2012 in the lowland rain forest (350 m asl) at the foothills of the south-west slope of Mt Cameroon.

Variables correlated	r	n	df	P
Max temperature and number of rainy days	−0.830	12	10	0.0008
Min temperature and number of rainy days	−0.593	12	10	0.0420
Max temperature and rainfall	−0.852	12	10	0.0004
Min temperature and rainfall	−0.524	12	10	0.0807

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Appendix 1

Souriau A, Kohoutová H, Reif J, **Vokurková J**, Petrusek A, Reifová R, Petrusková T (2018) Can mixed singing facilitate coexistence of closely related nightingale species? *Behavioral Ecology*, 29, 925–932.

Behavioral Ecology

The official journal of the ISBE International Society for Behavioral Ecology

Behavioral Ecology (2018), 29(4), 925–932. doi:10.1093/beheco/ary053

Original Article

Can mixed singing facilitate coexistence of closely related nightingale species?

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Received 7 September 2017; revised 14 February 2018; editorial decision 9 March 2018; accepted 23 March 2018; Advance Access publication 18 April 2018.

Knowledge of the mechanisms facilitating the coexistence of closely related competing species is crucial for understanding biodiversity patterns. The concept of convergent agonistic character displacement (ACD) suggests that interspecific interference competition may lead to convergence in territorial signals between species, which helps to establish interspecific territoriality and thus facilitate the species coexistence. Despite a strong theoretical background, however, empirical evidence for convergent ACD in nature is still scarce. Here we tested whether mixed singing (i.e. copying of elements from songs of a closely related sympatric species) in the Thrush Nightingale (*Luscinia luscinia*) in a secondary contact zone with the Common Nightingale (*L. megarhynchos*) could represent a case of convergent ACD. Using playback experiments, we measured the intensity of physical and vocal territorial responses of Common Nightingale males to 3 stimuli: conspecific song, pure heterospecific song, and mixed heterospecific song of the Thrush Nightingale. We found that Common Nightingale males showed a stronger physical territorial response to conspecific than both pure and mixed heterospecific songs. However, the intensity of vocal territorial response significantly increased with the presence of Common Nightingale elements in the stimuli, being lowest to pure heterospecific songs, intermediate to mixed heterospecific songs, and strongest to conspecific songs. These results indicate that mixed singing in the Thrush Nightingale may indeed be a case of convergent ACD. Our findings highlight the potential importance of mixed singing in facilitating species coexistence in the early stages of divergence.

Key words: **agonistic character displacement, bird song, mixed singing, interspecific competition, territoriality, vocal mimicry.**

INTRODUCTION

Interspecific interactions—including competition over resources, aggressive behavior, and hybridization—very often result in the phenotypic divergence of species in sympatry known as character displacement (Brown and Wilson 1956). Such divergence is assumed to be adaptive in terms of reducing the intensity of ecological competition (ecological character displacement; Pfennig and Pfennig 2009), avoiding misdirected aggression (divergent agonistic character displacement, hereafter ACD; Grant and Grant 2006; Grether et al. 2009), or decreasing the cost of hybridization (reproductive character displacement or reinforcement; Dobzhansky 1937; Butlin 1989). An apparent exception to this pattern concerns agonistic territorial signals involved in interference competition, which, compared to most other traits, relatively often converge between species in sympatry (Cody 1969; Brown 1977; Tobias et al. 2014). It has been suggested that convergence in these signals could be adaptive in terms of facilitating the communication between species and the establishment of interspecific territoriality. This would allow animals to resolve territorial disputes without direct physical conflict and avoid resource competition by forcing competitors from their territories. As a consequence, convergence in agonistic territorial signals can reduce both the costs of aggressive physical conflicts and the intensity of competition over resources between species, allowing species with close ecological requirements to coexist (Brown 1977; Sorjonen 1986; Tobias and Seddon 2009; Gil 2010; Reif et al. 2015). Such convergence in agonistic signals has been called convergent ACD (Grether et al. 2009, 2017).

Bird song is an important agonistic territorial signal that helps birds defend their territories without resorting to harmful physical attacks. It has been repeatedly demonstrated that bird song may converge in sympatric species, both in suboscine birds whose song is genetically determined (Tobias and Seddon 2009; Tobias et al. 2014)

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and in oscine birds whose song is transmitted culturally (Slater 1989; Catchpole and Slater 2008). Convergence in song may be caused by shifts in various song characteristics (e.g. temporal or syntax parameters; Secondi et al. 2003). However, in oscine birds this convergence is often caused by heterospecific song learning. This typically leads to a phenomenon known as mixed singing when males of one species incorporate song elements of another closely related species in their repertoires (Helb et al. 1985; Haavie et al. 2004; Gorissen et al. 2006; Vokurková et al. 2013). Mixed singing has long been assumed to result from erroneous heterospecific learning that brings no advantage to birds (Helb et al. 1985) or could even be maladaptive as it increases the likelihood of hybridization (Qvarnström et al. 2006). However, some indications suggest that it might be adaptive and represent a case of convergent ACD (Gorissen et al. 2006; Reif et al. 2015), although direct evidence is still missing. In this context, mixed singing may be a good example of vocal mimicry as defined by Dalziell et al. (2015), where a change in vocalization causing vocal resemblance between species confers a selective advantage to the mimic.

An important criterion for demonstrating convergent ACD (Grether et al. 2009; Tobias and Seddon 2009) is to show that convergence in a territorial signal increases the intensity of the territorial response towards heterospecifics. We tested this prediction in 2 closely related oscine species, the Common Nightingale (*Luscinia megarhynchos*) and the Thrush Nightingale (*L. luscinia*), which show song convergence in sympatric areas. These species diverged about 1.8 Mya (Storchová et al. 2010) and currently co-occur in a secondary contact zone spreading from north-eastern Germany to the Black Sea (Sorjonen 1986), where they occasionally hybridize (Becker 2007; Kverek et al. 2008; Reifová et al. 2011a; Mo kovský et al. 2018). The 2 species have distinct vocalizations but otherwise are very similar morphologically (with only minor differences in size and the intensity of plumage coloration) as well as ecologically (Cramp and Perrins 1994), which leads to aggressive territorial interactions (Reif et al. 2015). Competition between the species has apparently resulted in partial habitat segregation in sympatry (Reif et al. 2018; Sottas et al. 2018) and ecological character displacement in beak morphology (Reifová et al. 2011b). Several studies also suggest that the Thrush Nightingale, which is slightly larger than the Common Nightingale, might be competitively dominant (Sorjonen 1986; Stadie 1991; Reifová et al. 2011b), although the levels of heterospecific aggression do not differ between the species (Reif et al. 2015).

Song convergence in this pair of nightingale species is asymmetric. Although the songs of the 2 species clearly differ in allopatry, Thrush Nightingale males from sympatric populations often incorporate songs of the Common Nightingale in their repertoire (Sorjonen 1986; Vokurková et al. 2013). The frequency of Thrush Nightingale mixed singers varies among different sympatric localities but can be over 70% in localities where both species breed close to each other (Vokurková et al. 2013). The proportion of heterospecific songs in the repertoires of mixed singers is highly variable among individuals; it often exceeds 50%, occasionally reaching up to 100% (Vokurková et al. 2013).

To investigate the potential role of Thrush Nightingale mixed singing in interspecific communication and in mediating territorial interactions with the Common Nightingale, we measured the intensity of physical and vocal territorial signals of Common Nightingale males exposed to 3 playback stimuli: conspecific song, pure heterospecific song, and mixed heterospecific song of the Thrush Nightingale. We predicted that if mixed singing has an influence on interspecific male interactions, it should trigger a higher level of aggressive response than pure heterospecific song. Our results are discussed in the context of the theory of convergent ACD.

METHODS

Study area

Common Nightingale males were tested at localities within the zone of sympatry in Poland, in an area of ca 20 × 30 km along the floodplains of rivers Prosna (sites: Chocz, Czołnochów, Lisewo, Ruda Komorska) and Warta (sites: Zagórów, L d, Pyzdry) in the Greater Poland Voivodeship (52.12°N, 17.78°E), where both species are common breeders. Individuals were typically found in dense vegetation near riverbanks, where males were commonly observed to defend territories in close range to either conspecific or heterospecific neighbors.

Song stimuli

Three types of playback stimuli were prepared for the experiment: conspecific stimuli from recordings of Common Nightingale males (CN), heterospecific pure stimuli from recordings of pure-singing Thrush Nightingale males (TN), and heterospecific mixed stimuli from songs of mixed-singing Thrush Nightingale males (Mix) (Figure 1). A previous study, from which our experimental design was derived (Reif et al. 2015), already demonstrated that neither Common nor Thrush Nightingale males show any response to playback of a noncompeting species' song; we thus did not include such control stimulus in the present experiment.

Both pure song categories (CN, TN) contained only species-specific song types (based on a prior exploration of local song repertoires and syntax, as well as on comparison with allopatric populations of either species). The mixed stimulus (Mix) was characterized by the presence of both Thrush Nightingale and Common Nightingale song types (identified by comparison with an extensive catalogue of Common Nightingale song types; Vokurková et al. 2013) within the song of one Thrush Nightingale individual, but could also include song types where typical Common Nightingale syllables occur within Thrush Nightingale song structures (Figure 1, Supplementary Figure 1).

The playback stimuli were derived from recordings from different localities in the same study area obtained in 2008 and 2015 in order to minimize potential familiarity with an individual in the same location. All song manipulations and sound analyses were performed using Avisoft-SASlab Pro version 5.2 (www.avisoft.com). For each stimulus category, we used 90-s long sections from 15 to 19 recordings containing active singing without long pauses (pause duration below 6.6 s), selected according to their signal quality (song rate by category (mean ± SD): TN 7.56 ± 1.30, CN 10.89 ± 1.48 and Mix 7.92 ± 1.47 song types/min). The number of stimuli for testing at each locality was constrained by the need to use songs originating in the region (and thus unlikely to be perceived as foreign) but at places distant enough to reduce the risk of potential previous experience of the tested birds with the same singer or repertoire. Thus, while the majority of stimuli were only used once in the experiments, some had to be re-used (number of use (mean ± SD): 1.53 ± 0.65).

All stimuli were edited to remove the background noise (including the songs of other species). Some recordings of Thrush Nightingale mixed songs were further edited by removing some songs in order to balance the ratio of each species' song types (proportion of Thrush Nightingale song types in stimuli (mean ± SD): 63.5 ± 19.5%).

The amplitude of the tracks was normalized to 50% of the dynamic range (with bias offset from the signal removed) in Avisoft-SASLab Pro. During playback, the volume was standardized

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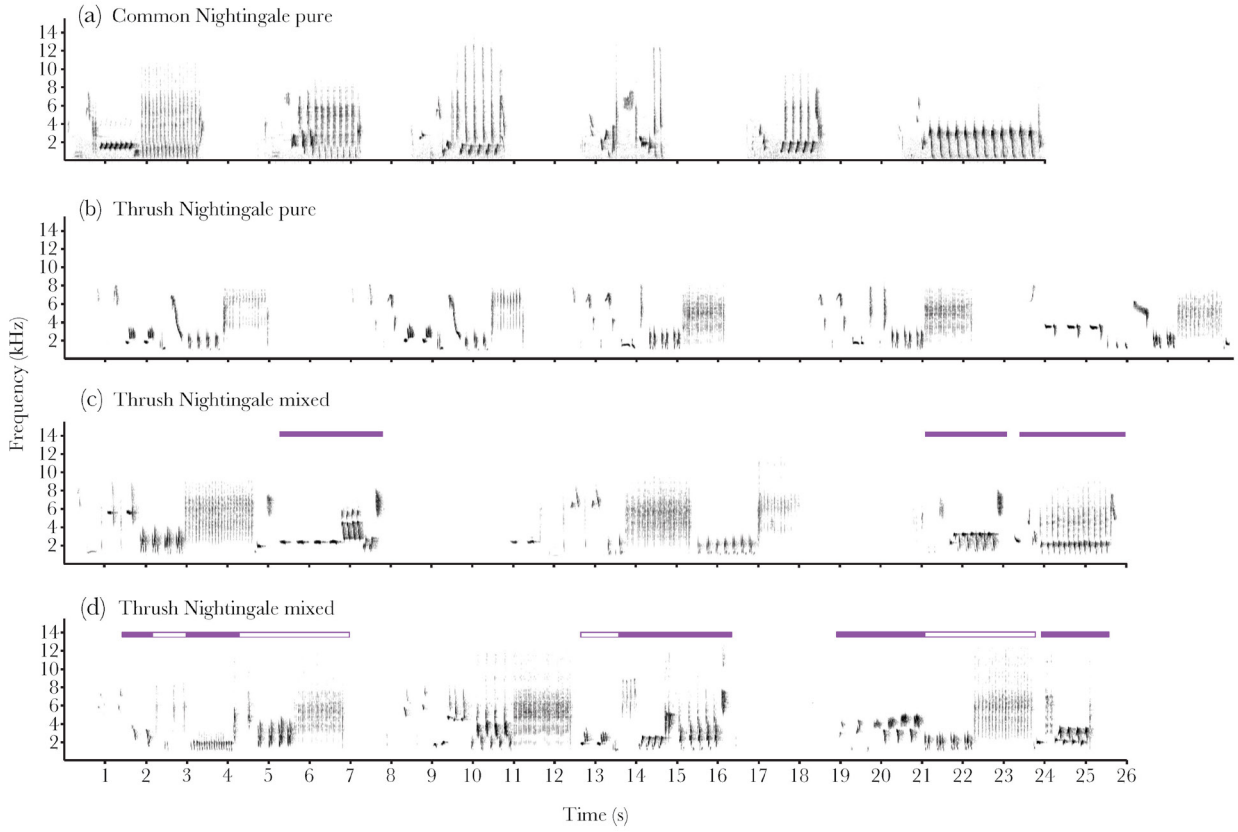


Figure 1
Spectrograms from samples of the 3 categories of stimuli: conspecific (Common Nightingale song; a), heterospecific pure (Thrush Nightingale song; b), and mixed (c, d). Mixed Thrush Nightingale songs may include either completely copied Common Nightingale song types (indicated by full horizontal bars), or song types in which syllables characteristic for song types of both species are mixed (striped horizontal bars). Songs are separated by pauses of silence, usually at least several seconds long, and consist of numerous syllables (which we define here as the invariant units within the vocalization, composed of one or multiple continuous traces in the spectrogram). Song types are then defined by a characteristic order of dominant syllables (Vokurková et al. 2013). Detailed spectrograms of selected song types are provided in Supplementary Figure 1.

according to local conditions to match the natural amplitude of a male singing from the same distance.

Experimental procedure

Playback experiments were conducted during the first 2 weeks of May 2016, soon after the return of the 2 species from their wintering grounds in Africa, as this is the most active period of territorial competition for males (Cramp and Perrins 1994; Reif et al. 2015). The tests were performed during daylight (from 0600 to 1900 h) in the absence of rain and strong wind, when the birds were spontaneously singing and most likely willing to react to intruders.

The experimental design generally followed Reif et al. (2015) with some modifications needed for the purposes of this study. We used a speaker (MIPRO MA-101) mounted on a tripod, on top of which a taxidermic dummy of either species was attached: Common Nightingale for conspecific playbacks and Thrush Nightingale for heterospecific playbacks. These visually very similar dummies (reflecting the close phenotypic similarity of both species) were used to provide a target allowing the tested bird to express a full range of behavioral responses (see Petrusková et al. 2008 for further discussion of the pros and cons of dummy use in playback experiments).

Vocal responses of the tested males were recorded using a digital recorder (Marantz PMD660) connected to a directional microphone (Sennheiser ME67), and direct behavioral observations were performed by a pair of observers (AS, HK) from a safe distance (ca. 15–20 m), to avoid potential disturbances by the presence of humans. The presence of a bird in its territory was assessed from both acoustic cues and visual observations. Each tested individual was presented with all 3 stimuli in a randomized order.

For the experiment, we chose actively singing territorial males that did not directly interact with their neighbors. The experimental device (loudspeaker with the taxidermic dummy) was set ca 1 m from the vegetation patch where the bird had been singing. Several minutes after the equipment set-up, the first stimulus was played for 3 min. Song and other behavioral reactions were recorded during the playback and 3 min after the playback ceased (to also include late responses). The same procedure was repeated with the remaining stimuli, each after a break of at least 1 h to avoid overstimulation and to clearly differentiate among stimuli. All 3 stimuli were tested on the same day, to minimize the chance of predation or territorial shifts between days. Directly neighboring birds were not tested on the same or subsequent days. Shortly after the experiment, the tested bird was lured by playback and captured

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by mist-netting to confirm the species identity, and to avoid any risk of repeated testing of the same individual in the future.

Evaluation of male territorial response to playback

Overall, we analyzed the responses of 27 Common Nightingale males. The full data set consisted of 21 males for all 3 types of stimuli; for 6 individuals, 1 stimulus had to be excluded (in 3 cases heterospecific pure stimulus, twice heterospecific mixed stimulus, once conspecific stimulus) due to a disturbance in experimental conditions (weather, intrusion by another male, etc.) or the absence of the bird in its territory for a given playback trial.

The territorial response of nightingale males to a simulated intrusion of the competitor into its territory (as already studied by Reif et al. 2015) typically begins with an expression of interest by approach, that is, an initial exploratory behavior within the vegetation cover, for example jumping to a closer perch soon after the playback starts. More intensive responses, ordered by increased aggressiveness (Reif et al. 2015), include the following: 1) flyover, that is, threatening the presumed intruder by a short flight from a nearby location (but more than 1 m away from the dummy); 2) jumping out of the vegetation cover to restlessly investigate the playback device from the ground (jumping, or "running" in Reif et al. 2015), 3) flight-attack, that is, flight directed towards the dummy with a very close approach (below 1 m), and 4) physical contact, that is, escalating aggression to direct physical attacks on the dummy. Furthermore, most males respond to playback by singing either during or soon after the playback stimulus.

The physical territorial response was monitored during the playback itself (3 min) and during the subsequent 3 min of the post-playback phase (i.e. 6 min in total). The responses were described by a set of behavioral categories (see Table 1) ranging from low to high level of aggressiveness as follows: jumping on the ground (count), flyovers (count), and direct physical contact (% of time attacking); no flight attacks were observed in the present experiments. In addition, we evaluated the strongest physical response shown by the bird during the experiment (as defined above, ranging from 1 = interest in the dummy without close approach, to 5 = physical attack) as an ordinal variable. The distance to the dummy was assessed throughout the experiment, as it is also expected to reflect different degrees of risk taken by the bird and its motivation to get involved in agonistic behavior (Searcy et al. 1997; Hyman et al. 2004; Reif et al. 2015), and the minimal estimated distance category was noted. Time spent by a tested individual within 4 different distance ranges from the dummy (<1 m, 1–3 m, 3–5 m, and >5 m) was also quantified.

Several song parameters (Table 1) were extracted from the recordings covering 6 min of both the playback and post-playback phases, based on variables already used in previous studies on *Luscinia* species (e.g. Kunc et al. 2005; Tur oková et al. 2011): mean song duration (in seconds), mean pause duration (the duration of silence between 2 consecutive songs, in seconds), song rate (mean number of songs per minute), and proportion of time spent by singing (% of time of bird presence).

Statistical analysis

Statistical analysis of the physical and vocal territorial responses of the tested males was performed with R software 3.2.2 (R Core Team 2014). We first used Principal Component Analysis (PCA) to summarize multiple song and behavioral variables measured during the experiments into independent factors suitable for further analyses. PCA was based on a correlation matrix of all 13 variables describing both physical and vocal responses (Table 1). It was run using the "principal" function in the "psych" package v. 1.5.8 (Revelle 2017) with a varimax rotation to maximize the variances of the squared normalized factor loadings across variables for each factor, thus facilitating interpretation of the resulting principal components.

Then, we used the first 2 principal components (i.e. those accounting for most of the variation in the data) as response variables in 2 linear mixed-effects models (one for each principal component) to test whether the responses differed between different types of stimuli. Besides song stimulus, each model contained a fixed-effect variable expressing the order of stimuli (from 1 to 3). This allowed us to control for a possible influence of habituation or overstimulation (e.g. Petrinovich and Patterson 1979; Petrusková et al. 2008; Tur oková et al. 2011) caused by presenting several stimuli to the same individual during the experiment. Individual identity was treated as a random effect in the models. Linear mixed-effects models were run with the "lme" function in the "nlme" package v. 3.1-122 (Pinheiro et al. 2017), and we used type III ANOVA from the "car" package v. 2.1-0 (Fox and Weisberg 2011) to test for the significance of respective variables.

Ethical note

No individual was hurt during our noninvasive experiments. Upon completion of the experiment, individuals were captured by

Table 1

Factor loadings obtained by principal component analysis on the responses of Common Nightingale and Thrush Nightingale males during playback experiments

Variable	Description	PC1	PC2
Song duration	mean duration of songs	−0.21	0.34
Pause duration	mean duration of pauses (shorter than 6.6 s)	−0.17	−0.84
Song rate	number of songs per minute	0.25	0.58
Singing time	% of time spent actively singing	−0.07	0.82
Jumping	excited jumping below the dummy	0.82	0.14
Flyovers	aggressive flying over the dummy	0.27	−0.06
Physical contact	time spent on the dummy, attacking	0.65	−0.19
Max aggressiveness	highest level on aggressiveness scale	0.92	0.07
Closest distance	minimal distance to dummy (m)	−0.59	−0.51
Time <1 m	time spent at distance 0 to 1 m	0.76	0.13
Time 1–3 m	time spent at distance 1 to 3 m	0.60	0.40
Time 3–5 m	time spent at distance 3 to 5 m	0.00	0.46
Time >5 m	time spent at distance over 5 m	−0.57	−0.49

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mist-netting with a conspecific playback lure and ringed for subsequent recognition. Manipulation with birds was approved by the Local Ethical Committee for Scientific Experiments on Animals in Poznań (permission no. 17/2015) and capturing the birds was performed under a license provided by the Gdansk Bird Ringing Centre (license no. 405/2051).

RESULTS

All tested males responded to the playback stimulation, irrespectively of the presented stimulus. In the vast majority of cases, reaction to playback started with a cautious approach to observe the "intruder," usually followed a moment later by actively counter-singing towards the speaker, before escalating to stronger physical territorial reactions in many cases. The first 2 principal components explained altogether 50.4% of the overall variance. The first PC axis (PC1, explaining 34.6% of the variance) was positively correlated with the intensity of the physical territorial response and the time spent closer than 3 m from the loudspeaker with the dummy. The second axis (PC2, explaining 15.8% of the variance) was positively correlated with the intensity of the vocal territorial response and the time spent at an intermediate distance, from 3 to 5 m (Figure 2).

According to the linear mixed-effects model, the physical territorial response of the tested males (reflected by PC1) significantly differed among playback stimuli ($^2 = 34.4$, $P = 3.4 \times 10^{-8}$, df = 2, 70), with a more aggressive response to conspecific stimulus than to both heterospecific ones, either pure or mixed (Figure 3a, Supplementary Table 1). We did not observe a significant difference between the mixed and pure heterospecific stimuli (Figure 3a). This outcome was in line with the general expectation of a more aggressive physical territorial response to conspecific than to heterospecific stimuli, being reflected by closer approaches to the device, more time spent on the ground investigating it, as well as the occurrence of physical attacks (which occurred in conspecific trials only).

The vocal territorial response of the tested birds (reflected by PC2) also significantly differed among stimuli ($^2 = 27.4$, $P = 1.1 \times 10^{-6}$, df = 2, 70). Specifically, the males exhibited a stronger reaction to the conspecific stimulus than to pure and mixed heterospecific stimuli (Figure 3b, Supplementary Table 1), demonstrated by more time spent singing, a higher song rate, longer song duration and accordingly shorter pauses in song bouts. In contrast to the physical response, however, the vocal response to the mixed heterospecific stimulus was significantly more intense than the response to the pure heterospecific stimulus (Figure 3b, Supplementary Table 1). The intensity of the response to the mixed heterospecific stimulus was thus intermediate between the responses to pure heterospecific and conspecific stimuli.

Although a habituation effect could be expected due to presentation of the respective stimuli to the same individual within a few hours, we found no significant effect of the order of the presented stimuli on either physical or vocal territorial responses (PC1: $^2 = 3.84$, $P = 0.15$, df = 2, 70; PC2: $^2 = 1.92$, $P = 0.38$, df = 2, 70).

DISCUSSION

Our playback experiments testing whether mixed singing of Thrush Nightingale males in the secondary contact zone with the Common

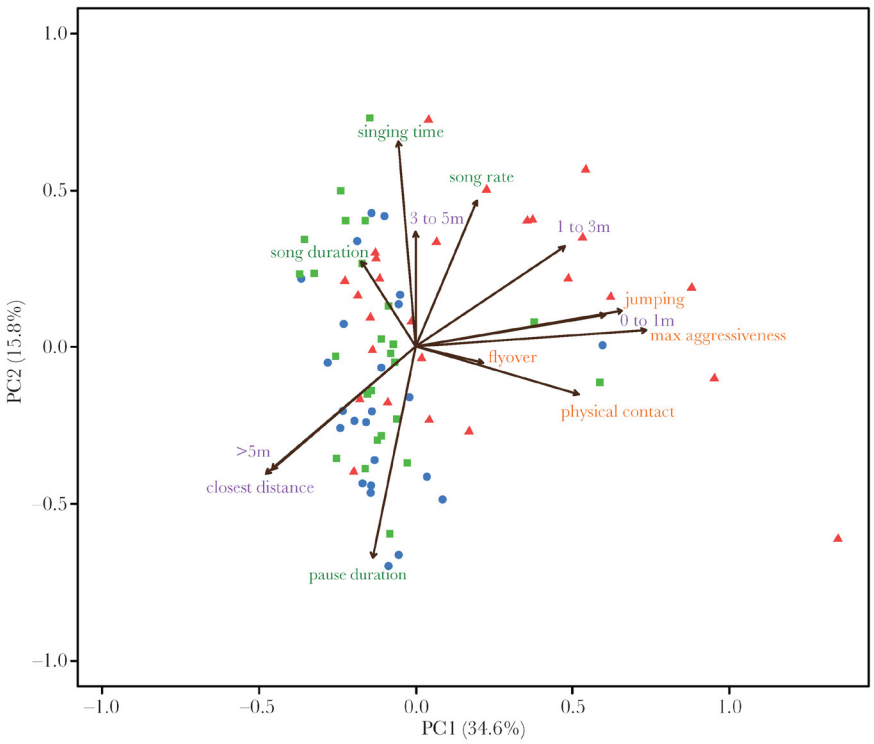


Figure 2
Principal Component Analysis (after normalized varimax rotation) based on the categories of distance (purple), singing characteristics (green) and physical aggressive responses (orange). The first 2 principal components explain 34.6% (first axis) and 15.8% (second axis) of the overall variation. The dots represent Common Nightingale individuals tested for the respective stimuli: conspecific (red triangle), mixed (green square), and pure heterospecific (blue circle).

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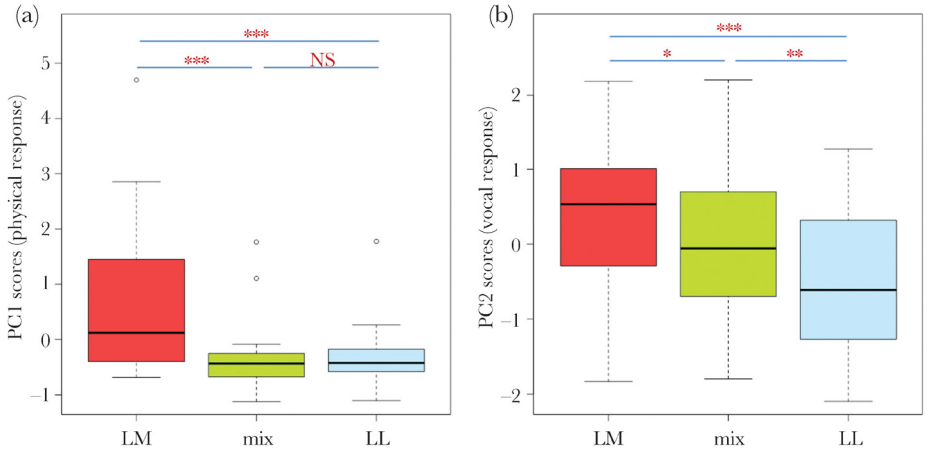


Figure 3
Mean scores for responses of Common Nightingale males to particular song stimuli (LL = Thrush Nightingale, LM = Common Nightingale, mix = mixed singing from Thrush Nightingale) during playback experiments estimated by a linear mixed-effects model run separately for PC1 corresponding to physical responses (a) and PC2 corresponding to vocal responses (b). Box plots depict the median, interquartile range, and range. (NS: $P > 0.05$, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$).

Nightingale affects the level of interspecific territoriality between the species resulted in 2 main findings. 1) Common Nightingale males discriminated between intruders singing conspecific and heterospecific songs, regardless of whether the heterospecific stimulus was pure or mixed. This was manifested by a stronger physical territorial response of the tested males to the conspecific stimulus than to both pure and mixed heterospecific stimuli. 2) However, the vocal territorial response was gradually more intense with an increasing presence of Common Nightingale elements in the stimuli, from a low response to pure heterospecific, an intermediate response to mixed heterospecific, to a strong reaction to conspecific songs. This suggests that the presence of Common Nightingale song characteristics in Thrush Nightingale songs triggers a higher level of the vocal territorial response in Common Nightingale males but does not affect the physical territorial response.

Our results support the view that mixed singing performed by Thrush Nightingale males has a role in between-species communication and can mediate interspecific territoriality. Below, we discuss our results in the context of convergent ACD (Grether et al. 2009). We argue that mixed singing in oscine birds might be adaptive in terms of facilitating the coexistence of closely related species, and thus fits the concept of vocal mimicry (Dalziell et al. 2015). However, we also mention possible alternative explanations of the presented results.

Grether et al. (2009) defined 8 criteria for demonstrating ACD. The phenotypic shift 1) should be at least partially genetically determined, 2) should be unlikely to have arisen by chance, 3) should represent a true evolutionary change and not the result of biased colonization or extinction events, 4) should persist after controlling for environmental variables other than the presence of the competing species, 5) is not likely to be a pleiotropic effect of another evolutionary process, and 6) is not a product of hybridization. In addition, there should be 7) evidence that the observed phenotypic shift affects the intensity of interspecific interference competition and 8) independent evidence for interspecific interference competition. Meeting all these criteria is quite difficult. Grether et al. (2009) reported 18 possible examples of convergent ACD across fishes, amphibians, reptiles, birds, and insects, including as many as 12 cases of convergence in bird vocal signals and vocal recognition. However, only one of these examples, concerning heterospecific aggressive behavior in the brook stickleback *Culaea inconstans* (Peiman and Robinson 2007), satisfied all 8 criteria for demonstrating ACD, whereas the rest of examples satisfied only 3 to 5 criteria. Based on the results presented in this study and previous studies, we have assembled evidence for 6 criteria, making our system one of the best-supported examples of convergent ACD.

Character displacement is typically demonstrated as a phenotypic shift between sympatric and allopatric populations (Brown and Wilson 1956). In the case of convergent ACD, this shift results in more similar phenotypes in sympatry than in allopatry. To show that this pattern is not caused by chance (criterion 2), it is desirable to show that the convergence occurs in multiple independent populations. In these 2 nightingale species, song convergence has been independently demonstrated in multiple parts of the secondary contact zone, including Poland (Sorjonen 1986; Vokurková et al. 2013), Germany (Becker 2007) and Hungary (Schmidt 1973). Moreover, the change in the Thrush Nightingale song cannot result from biased colonization or extinction (criterion 3), as no mixed singers have been observed in allopatry (Vokurková et al. 2013). It is also highly unlikely that the mixed song in the Thrush Nightingale represents an adaptation to the local acoustic environment (criterion 4), as both species occupy very similar habitats in sympatry and allopatry (Kuczyński and Chylarecki 2012), and if anything there is a tendency to habitat divergence rather than convergence in sympatry (Reif et al. 2018). The effect of hybridization (criterion 6) has also been ruled out as a possible cause of mixed singing in nightingales (Vokurková et al. 2013). Finally, in this study we provide evidence that the observed phenotypic shift affects the intensity of interspecific interference competition (criterion 7), which complements the previously provided evidence for interspecific interference competition between both nightingale species (criterion 8) (Reif et al. 2015).

One strong argument against the possible adaptiveness of mixed singing concerns the fact that in oscine birds, the song is not genetically determined (criterion 1), but culturally learned (Catchpole and Slater 2008). In this situation, mixed singing does not need to

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be a heritable and thus evolvable trait. However, new studies on the genetic basis of the song learning process have shown that although song is learned in oscine birds, there is a genetically determined ability to discriminate the species-specific song, which is then preferentially learned (Scharff and Adam 2013; Wheatcroft and Qvarnström 2017). This genetically determined song discrimination can play an important role in maintaining reproductive isolation between closely related species by reducing the probability of heterospecific song learning (Wheatcroft and Qvarnström 2017). Nevertheless, mutations in genes for song discrimination could cause the bird to learn not only conspecific but also heterospecific songs. Mixed singing might thus in principle have a genetic basis. However, to prove this in our system, and demonstrate that mixed singing in the Thrush Nightingale evolved to reduce the level of interspecific competition, it would be necessary to show that the ability (or tendency) to learn heterospecific songs is higher in sympatric than in allopatric populations.

According to criterion 5, a pleiotropic effect of another evolutionary process should be ruled out. Vokurková et al. (2013) speculated that mixed singing might possibly be the result of sexual selection rather than selected to reduce interspecific competition. Without the knowledge whether Thrush Nightingale females respond differently to pure and mixed song, it is difficult to distinguish between these alternatives. However, we consider the explanation based on sexual selection alone unlikely, given that a preference of Thrush Nightingale females for Common Nightingale song would lead to increased interspecific hybridization, as has been observed in *Ficedula* flycatchers (Qvarnström et al. 2006).

It appears surprising that mixed singing is so common in the Thrush Nightingale but has not been observed in the Common Nightingale. Copying of song types from conspecific neighboring males, which facilitates territorial interactions, has been documented within populations of both species (Hultsch and Todt 1981; Naguib and Todt 1998), and experiments in captivity demonstrated that both are able to learn the heterospecific song (Stadie 1983). Nevertheless, the reaction to heterospecific songs in sympatry differs between species: in playback experiments, the Thrush Nightingale males reacted with the same intensity to conspecific songs and to heterospecific songs of the Common Nightingale, while Common Nightingale males reacted to heterospecific songs more weakly (Reif et al. 2015). This difference, however, may be a direct consequence of mixed singing. Some Thrush Nightingale males in sympatry seem to compose their song entirely from Common Nightingale song types (Vokurková et al. 2013), and their conspecifics should thus react to the Common Nightingale song as to their own.

According to the physical territorial response in our experiments, Common Nightingale males were clearly able to distinguish between conspecific and heterospecific intruders, no matter whether the latter sing pure or mixed songs. By contrast, the vocal territorial response of Common Nightingale males was strongest for the conspecific stimuli, less strong for the mixed heterospecific stimuli and weakest for the pure heterospecific stimuli. The differences in responses to conspecific and heterospecific stimuli are unlikely to have been caused by accompanying taxidermic dummies. The tested males usually did not approach closer than 5 m from the dummy in heterospecific trials, and thus were unlikely to distinguish the minor species-specific differences between the Common and Thrush Nightingale dummies. Thus, we presume that the male responses were triggered by the acoustic stimuli alone, not only when comparing pure and mixed heterospecific song (accompanied by the same Thrush Nightingale dummy) but also when comparing the conspecific and heterospecific stimuli. This is also consistent with the species' biology: singing interactions of nightingales usually happen from a large distance out of sight range, often at night (Hultsch and Todt 1981; Griessmann and Naguib 2002), and therefore play a key role in their territoriality.

Our findings, together with other recent works on the nightingale system (Vokurková et al. 2013; Reif et al. 2015), support the view that mixed singing performed by Thrush Nightingale males in the secondary contact zone with the Common Nightingale may represent a case of convergent ACD, and, in a broader sense, a prominent case of vocal mimicry. However, further work on the genetic basis of song discrimination in sympatric and allopatric populations of the Thrush Nightingale as well as studies demonstrating that mixed singing increases the fitness of the sympatric Thrush Nightingale males would be needed to confirm the adaptive role of mixed singing in facilitating the coexistence of these closely related species before divergence in ecological niches evolves.

FUNDING

The study was supported by grants of the Czech Science Foundation (grant numbers 15-10884Y, 18-14325S) to R.R.

We wish to thank T. Albrecht, B. Cramer, P. Dolata, P. Kverek, K. Opletalová, and C. Sottas for field assistance and 2 anonymous reviewers for their constructive comments on earlier versions of this paper. T. Osiejuk kindly helped us with obtaining permissions for research.

Authors' Contributions: R.R., T.P., A.P., and J.R. conceived the idea; A.S., J.R., T.P., A.P., and R.R. designed the research; A.S., H.K., J.V., and J.R. collected data; A.S., H.K., T.P., A.P., and J.R. analyzed data; A.S., R.R., and T.P. drafted the manuscript with inputs from all authors. All authors gave final approval for publication.

Conflict of Interest: The authors declare no competing interests.

Data accessibility: Analyses reported in this article can be reproduced using the data provided in Souriau et al. (2018).

Handling editor: Naomi Langmore

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Appendix 2

Reif J, Jiran M, Reifová R, **Vokurková J,** Dolata P, Petrusek A, Petrusková T (2015) Interspecific territoriality in two songbird species: potential role of song convergence in male aggressive interactions, *Animal Behaviour*, 104, 131–136.

Animal Behaviour 104 (2015) 131–136

Contents lists available at ScienceDirect

Animal Behaviour

journal homepage: www.elsevier.com/locate/anbehav

Interspecific territoriality in two songbird species: potential role of song convergence in male aggressive interactions

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ARTICLE INFO

Article history:
Received 4 December 2014
Initial acceptance 16 January 2015
Final acceptance 6 March 2015
Published online
MS. number: 14-00982R

Keywords:
aggression
agonistic character displacement
animal signalling
birds
interference competition
interspecific interactions
song
territoriality

In animals, interspecific interference competition is often associated with their aggressive behaviour. The intensity of interspecific aggression and the outcomes of interference competition between closely related species might be substantially modified by copying of vocal signals used in territory defence. Here we tested the hypothesis that song convergence observed in a secondary contact zone of two songbird species, the common nightingale, *Luscinia megarhynchos*, and the thrush nightingale, *Luscinia luscinia*, might be an adaptive response to interspecific interference competition. These species are morphologically and ecologically very similar. However, the thrush nightingale is slightly larger and several lines of evidence indicate its competitive dominance. In the secondary contact zone most thrush nightingales incorporate common nightingale song types in their repertoires. Using playback experiments, we evaluated the strength of nonvocal aggressive responses of both species to conspecific and heterospecific stimuli. The species did not differ in aggressive responses to a heterospecific stimulus, suggesting that competitive dominance is not associated with higher interspecific aggressiveness in nightingales. Interestingly, while the common nightingale reacted significantly more aggressively to the conspecific than the heterospecific stimulus, the thrush nightingale showed similarly strong responses to both stimuli. We suggest that this similar level of interspecific and conspecific aggression in the thrush nightingale results from mixed singing of this species in sympatry, as males may not distinguish conspecifics from heterospecifics by song alone. Our results are consistent with the concept of convergent agonistic character displacement. According to this theory, vocal convergence might be adaptive in species that overlap broadly in resource use, as it leads to better distinction of territory boundaries between the species and thus reduces the level of interspecific competition.
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Aggressive behaviour connected with territory defence is the predominant form of interference competition in animals (Cody, 1974; Langkilde & Shine, 2004; Robinson & Terborgh, 1995) with important ecological consequences (Pigot & Tobias, 2013). It can occur between individuals of the same species as well as between individuals belonging to different species. Ecological and evolutionary aspects of interspecific aggression are, however, understudied compared to other interspecific interactions (Grether et al., 2013).

In some animal groups including birds, acoustic signals facilitate territory defence besides their function in mate choice and changes in such signals can have important consequences for aggressive interactions (Maynard Smith & Harper, 2003). Such changes often occur in secondary contact zones of closely related species (reviewed in Grether, Losin, Anderson, & Okamoto, 2009). It is often assumed that divergence in vocalization will evolve after secondary contact to minimize hybridization (Kirschel, Blumstein, & Smith, 2009; Sætre et al., 1997; Seddon, 2005). Convergence of acoustic signals in secondary contact zones, however, is also frequent, especially in birds (Haavie et al., 2004; Helb, Dowsett–Lemaire, Bergmann, & Conrads, 1985; Lemaire, 1977). This is probably because in many birds, particularly in passerines, vocalization is not genetically determined but learned through an imprinting-like process (Price, 2008). Misdirected imprinting and heterospecific

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http://dx.doi.org/10.1016/j.anbehav.2015.03.016
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learning can lead to transfer of vocal characters from one species to another resulting in song convergence in sympatry (Dalziell, Welbergen, Igic, & Magrath, 2014; Haavie et al., 2004; Helb et al., 1985). Such song convergence is usually considered to be maladaptive, as it can increase the rate of interspecific hybridization (Qvarnström, Haavie, Sæther, Eriksson, & Pärt, 2006). However, according to the theory of agonistic character displacement, song convergence can be positively selected in species that overlap broadly in resource use as it leads to better distinction of territory boundaries between the species and thus reduces the level of interspecific competition (Cody, 1969; Grether et al., 2009). Empirical evidence for such convergent agonistic character displacement, however, is so far scarce (Tobias & Seddon, 2009).

Two closely related songbird species, the common nightingale, *Luscinia megarhynchos*, and the thrush nightingale, *Luscinia luscinia*, represent a particularly interesting system to study how convergence in vocal signals affects competitive interactions and the speciation process. These species diverged approximately 1.8 million years ago (Storchová, Reif, & Nachman, 2010), and their ranges came into secondary contact with a narrow zone of sympatric occurrence in central and eastern Europe (Cramp, 1988), where they occasionally hybridize (Becker, 2007; Kverek, Storchová, Reif, & Nachman, 2008; Reifová, Kverek, & Reif, 2011). The species are morphologically very similar, but some distinctive species-specific diagnostic traits exist in body size, coloration and plumage (Svensson, 1992). The common nightingale and the thrush nightingale prefer the same dense shrubby habitats, mostly on moist sites (Cramp, 1988), and their territories are often in close proximity (Becker, 1995). Interspecific competition between these species can be inferred from spatially exclusive territories in sympatry (Sorjonen, 1986) and ecological character displacement in beak morphology (Reifová, Reif, Antczak, & Nachman, 2011). Several lines of evidence suggest that the thrush nightingale, which is slightly larger than the common nightingale, is competitively more dominant (Reifová, Reif, et al., 2011; Stadie, 1991). In sympatry, asymmetrical convergence in vocalization occurs: most thrush nightingales are mixed singers, incorporating common nightingale song types in their repertoires (Becker, 2007; Lille, 1988; Vokurková et al., 2013). The convergence is not caused by genetic introgression, but rather by heterospecific learning (Vokurková et al., 2013). This, however, does not mean that the tendency to asymmetric heterospecific learning cannot be genetically determined (Grether et al., 2009). The interesting question thus remains whether the mixed singing in the thrush nightingale is adaptive in terms of improving competitor recognition, increasing interspecific territoriality and reducing the intensity of interspecific competition.

In this study, we performed playback experiments to study the intensity of aggressive reaction towards conspecific and heterospecific males in both nightingale species in sympatry. Similar experiments on these species have been performed by Sorjonen (1986). However, Sorjonen (1986) only evaluated whether or not tested males reacted to the stimuli without quantification of reaction strength. Moreover, his methodology could have introduced substantial bias in the tested birds' responses owing to the nonindependence of the stimuli presented, and probably also by reuse of the same playback stimuli across experiments, resulting in pseudoreplication (Kroodsma, Byers, Goodale, Johnson, & Liu, 2001).

Here we conducted playback experiments testing the response of males of both species in a sympatric zone in a rigorous design overcoming the methodological problems mentioned above. We focused on the following questions. First, does the thrush nightingale, as a larger and presumably competitively dominant species, show a stronger response to the heterospecific stimuli than the common nightingale? Second, does the mixed singing of the thrush nightingale affect the intensity of its interspecific aggressive reaction? Based on the current knowledge of agonistic character displacement (Grether et al., 2009), we expected the strength of the thrush nightingale response to be similar to conspecific and heterospecific stimuli, while a stronger response to the conspecific than to the heterospecific stimulus was expected in the common nightingale.

METHODS

Study Area

The study was conducted in a ca. 200 km^2 area near the towns of Pleszew and Zagorow in central Poland (Wielkopolska region; 52°01′N, 17°48′E), in a central area of sympatry where both species occur in similar densities. In this region, nightingales were studied in floodplains of the rivers Prosna and Warta providing ample suitable habitats. Both species were present at the same sites, and in most cases we did not observe any habitat segregation. Territories of individual males did not overlap, and were often adjacent to each other irrespective of species identity.

Recording of Songs used as Playback Stimuli

Songs used for experiments were recorded in two areas of close allopatry: in the Czech Republic in 2007–2010 (common nightingale) and in northeastern Poland in 2009 (thrush nightingale). These allopatric areas were located 300 km and 350 km, respectively, from the sympatric study area and previous analyses confirmed that none of the individuals from these localities showed interspecific copying of song (Vokurková et al., 2013). As a control stimulus, we used songs of the willow warbler, *Phylloscopus trochilus*, a species occurring in high densities on the study sites among breeding nightingales. From each recording of an individual of a given species, a unique 150 s interval of good quality was cleaned from the background noise in the software Avisoft SASLab Pro version 5.2 (Specht, 2007) and then duplicated to create a 5 min long playback recording. In total, we had 58 of these 5 min recordings available for the experiments.

Experimental Design

The experiments were conducted in May 2011–2013, soon after the arrival of both species to their breeding sites, always within a 2-week period at the beginning of the breeding season to cover the period of the highest territorial activity of both species. Weather permitting; we worked in the morning (0600–1000 hours) and late afternoon (1600–2000 hours) when nightingales show high singing activity.

The general design of the experiment followed that of Turčoková, Pavel, Chutný, Petrusek, and Petrusková (2011) and was based on presenting playback songs of the focal species together with a corresponding taxidermic dummy (i.e. a specific dummy for each species). The taxidermic dummies were supplied by the Department of Zoology, Charles University in Prague, and showed all species-specific diagnostic traits (see Svensson, 1992). We first checked for individual territories by walking through the breeding sites and registering individual nightingales and their singing posts for several days at the beginning of the nightingale breeding season. Before each experiment, we first detected the position of an individual selected for the experiment to confirm its presence in the territory. Then we placed a loudspeaker MIPRO MA-101 and a taxidermic dummy (of the species corresponding to the playback stimulus) into a territory of the target individual near its singing post. Once the focal male was detected visually or acoustically in its

territory, we played the song of either the common nightingale or the thrush nightingale for 5 min. The observers were hidden ca. 20 m from the loudspeaker and dummy, to avoid any influence of human presence on the bird's behaviour. We noted the behaviour of the tested male and recorded its vocal activity (using the Marantz PMD 660 recorder and Sennheiser ME 67 shotgun microphone) for 5 min during the playback. After this session, the first part of the experiment was finished.

We presented the stimuli in a random order to avoid possible order effects. To avoid habituation and interaction between subsequent stimuli, the tested male was exposed to the stimulus of the second nightingale species (with the same design) a day after we conducted the first experiment, at approximately the same time of day and in the same fashion. On the same day, we never tested individuals occupying territories situated within hearing distance of the bird exposed to playback. All experiments were conducted by M.J. (playbacks and recording songs of tested individuals) and J.R. (observing and noting behavioural activity).

The control stimulus (represented by the willow warbler) was presented a day after the second part of the experiment to some of the tested birds each year of the study. However, we never observed any response to this type of stimulus. Therefore, we performed the experiment with the control with nine birds only to show that the lack of response is consistent (see below). Reducing the number of experiments with the control stimulus allowed us to maximize the number of experiments with conspecific and heterospecific nightingale stimuli during the short period when males are willing to respond to the playback, as, in many species, males cease aggressive responses towards an intruder after their mates start incubation (e.g. Nowicki, Searcy, Krueger, & Hughes, 2002).

Data Analysis

In total, we tested 15 common nightingale and 15 thrush nightingale individuals with both conspecific and heterospecific stimuli. Data from presentations of the control stimulus were obtained from five common nightingales and four thrush nightingales.

The behavioural response was recorded during the 5 min period when the playback was presented. Our description of the behavioural response is generally based on approach measures because approaching the rival has been found to be a key component of aggressive behaviour in both species (Stadie, 1991). Although some specific song patterns also play a role in aggressive interactions in the common nightingale (e.g. Schmidt, Kunc, Amrhein, & Naguib, 2008), it would be difficult to apply them to the thrush nightingale, as the song structure of the latter species has been much less studied. We categorized responses as follows (adapted from Petrusková, Petrusek, Pavel, & Fuchs, 2007): approach (individual approaches close to the dummy suggesting an interest in it; the approach is repeated or prolonged, not caused by accidental movement of the tested bird), flyover (flight over or behind the dummy, at a distance of more than 1 m), running (excited running on the ground below the dummy), flight-attack (flight directed at the dummy, within 1 m, but not resulting in physical contact), physical contact (attack on the dummy; individual typically sitting on its back and pecking).

These behavioural categories were mutually exclusive (i.e. an individual could not show behaviour of more than one category at the same time), and for each individual we calculated the proportion of time spent performing the behaviour in each category during an experiment. In one experiment, the sum of these proportions equals the time when a given individual showed any response to a given stimulus. We also calculated the percentage of time spent singing during the experiment. This activity was recorded independently of the five behavioural categories (see above); and we added the percentage of time singing to the analysis of the behavioural response.

All six variables summarizing the time investment in the five categories of nonvocal behavioural response (as defined above) and singing activity were subjected to a principal component analysis (PCA) because some variables were closely correlated with each other and thus were not independent (Table 1). The PCA was done on a correlation matrix of the arcsine-transformed variables (all variables were proportions). By means of PCA, we obtained new independent variables (PC axes) describing the most important gradients in the responses of tested individuals. The first two PC axes, which accounted for the largest part of the variability in nightingale responses (see Results), were taken as response variables for further analyses. We also estimated the minimum distance (m) to the dummy during the playback, and used it as an additional response variable in statistical analyses.

To test the differences between species in responses to the respective stimuli, we employed linear mixed-effects models relating the first PC axis, the second PC axis and the minimum distance to the dummy as the respective response variables to fixed effects of species (categorical variable with two levels: common nightingale and thrush nightingale), stimulus (categorical variable with two levels: conspecific and heterospecific) and their interaction. Although we presented the stimuli in random order (see above), we included the information about the order of a given stimulus presented (i.e. first or second) as a covariate to control for a possible order effect in the data (Petrusková, Petrusek, Pavel, & Fuchs, 2008). Since we presented two stimuli to each individual tested, we included the identification code of each individual as a categorical variable with random effects (as in Farwell & Marzluff, 2013).

The nightingale males never showed any interest during the playback of the willow warbler control stimulus (consistent with observations of Sorjonen (1986) who used the song of the bluethroat, *Luscinia svecica*, as a control). Thus, we did not include experiments with the control stimulus in the PCA and further analyses. Rather, we compared proportional time spent in aggressive responses (sum of all behavioural categories together, see above) between the control (willow warbler) and congeneric (i.e. the heterospecific nightingale) stimulus for each species separately by the linear mixed-effects model. The model contained the fixed-effect stimulus and order and the random effect of nightingale individual. The test confirmed a highly significant difference in the time spent in aggressive responses between the congeneric and control stimulus for both tested species: common nightingale – congeneric stimulus: mean = 95.20% of time spent in aggressive responses (SE = 0.91), control stimulus: mean = 0.00% (SE = 0.91), $F_{1,2} = 4811$, $P < 0.001$; thrush nightingale – congeneric stimulus: mean = 97.40% (SE = 1.09), control stimulus: mean = 0.00% (SE = 1.09), $F_{1,2} = 3550$, $P < 0.001$.

Ethical Note

Our study involved field observations of aggressive behaviour of 30 birds in response to two 5 min long playbacks. After the playback experiments, individual birds were caught, ringed and released by P.T.D. (ringing licence no. 55 and 61). Trapping and ringing were done in accord with The Nature Protection Act (Disposition no. 627 from 2013) in Poland. No birds were invasively sampled. Our research did not require approval by the Local Ethical Commission because the playback experiments do not fall within its authority in Poland according to The Act on Experiments on Animals (Disposition no. 289 from 2005). The playback was kept as short as possible to obtain data for the purposes of the current

Table 1
Variables describing behavioural response of males of the common nightingale and the thrush nightingale during playback experiments

Variable	Description	PC1	PC2	PC3	PC4
Approach	Approach to the dummy with signs of interest	−0.98	−0.15	0.13	0.01
Flyover	Flight over or behind the dummy	−0.05	0.13	−0.10	0.38
Running	Excited running below the dummy	0.15	0.01	−0.04	0.95
Flight-attack	Flight directed at the dummy (within 1 m)	0.00	−0.03	−0.04	0.35
Physical contact	Attack on the dummy	0.76	−0.25	0.60	0.00
Singing	Percentage of time spent singing	−0.34	0.90	0.27	0.00

The behavioural response was characterized by five variables represented by the mutually exclusive categories of behaviour (ordered by increasing aggressiveness from approach to physical contact) and one additional variable represented by singing. All six variables were subject to a principal component analysis. Relations of these variables to the four most important principal components (PC) are shown.

study and we are not aware of any consequences for subjects' breeding or welfare.

RESULTS

All males showed at least an interest in or, often, a stronger level of behavioural response to both conspecific and heterospecific playbacks. In other words, all individuals approached the dummy when exposed to the playback and 'approach' was also the behavioural category taking the highest proportion of time during the experiment (75% in the common nightingale and 85% in the thrush nightingale). Physical attacks on the dummy in response to the playback were also observed in both species. These attacks were more frequent among common nightingales (eight of 15 males attacked the dummy at least once) than thrush nightingales (five of 15 males).

The results of the PCA showed that the first PC axis (eigenvalue = 2.77) explained over 73.5% of the variability in nightingale responses to conspecific and heterospecific stimuli and was the only axis with an eigenvalue >1. This axis showed a gradient of increasing aggressiveness from weak responses represented by approaching to strong responses represented by physical attack and, to lesser extent, by running on the ground close to the dummy (Table 1, Appendix Fig. 1). The second PC axis (eigenvalue = 0.20) expressed the increasing proportion of time devoted to singing (Table 1, Appendix Fig. 1) but explained only 16.4% of the variability in responses. The remaining two axes did not provide a clear interpretation (Table 1) and accounted for a negligible part of the variability in responses (PC3: eigenvalue = 0.11, variability explained = 9.7%; PC4 eigenvalue = 0.00, variability explained = 0.2%).

The mixed-effects model performed with the first PC axis as a response variable showed that common nightingales generally responded more aggressively than thrush nightingales, irrespective of the stimulus (Table 2). Overall, the tested males (irrespective of species) were not more aggressive to the conspecific than to the heterospecific stimulus (Table 2). However, a significant interaction between species and stimulus indicates that responses to the two types of stimuli differed between the species (Table 2). The order effect was significant indicating that the stimuli presented first provoked a more aggressive response than the stimuli presented afterwards (Table 2). The order of stimuli was thus kept in the model to control for its effect.

The interpretation of the interaction between species and stimulus was elucidated by the analysis of contrasts (Fig. 1). The species did not differ in overall aggressiveness in response to the heterospecific stimulus ($t = 0.10$, $P = 0.923$), but the common nightingale was more aggressive in response to the conspecific stimulus than the thrush nightingale ($t = 2.12$, $P = 0.043$). The common nightingale responded significantly more aggressively to the conspecific than to the heterospecific stimulus ($t = 2.20$, $P = 0.036$; Fig. 1), but no difference was observed in the thrush nightingale ($t = -0.80$, $P = 0.432$; Fig. 1).

The mixed-effect models run for the second PC axis and for the minimum distance to the dummy as respective response variables did not find significant effects of any predictors (Table 2).

DISCUSSION

Our experiments, which quantified behavioural responses of two nightingale species to conspecific and heterospecific playbacks, revealed three important patterns. (1) Males of both species showed aggressive responses to each other's song (accompanied with a species-specific taxidermic dummy) but did not react to control stimuli. (2) The species did not differ in the level of aggressiveness in response to the heterospecific stimulus. (3) The common nightingale reacted significantly more aggressively to the conspecific stimulus than to the heterospecific stimulus, whereas the response of the thrush nightingale did not differ between stimuli.

The first pattern confirms the presence of interspecific aggression between nightingale species in sympatry already described by Sorjonen (1986). Individuals of both species were apparently excited by heterospecific playbacks and all tested birds approached the dummy in response to the playback. Moreover, physical attacks on the dummy were observed in heterospecific playback experiments in both nightingale species, although not in all individuals. This character of behavioural responses to simulated playback observed in nightingales is comparable to reactions observed in other passerines defending their territories such as *Phoenicurus* redstarts (Sedláček, Fuchs, & Exnerová, 2004) or *Acrocephalus* warblers (Catchpole, 1978). Moreover, the nightingales' congener, the bluethroat, which also boasts a highly complex song, showed similar levels of aggressive behaviour in response to playbacks of its different subspecies (Turčoková et al., 2011). Our demonstration of

Table 2
The effects of the type of stimulus, order of stimuli and species identity on the strength of aggressive response of the common nightingale and the thrush nightingale during playback experiments

Predictor variable	PC1			PC2			Minimum distance		
	df	*F*	*P*	*df*	*F*	*P*	*df*	*F*	*P*
Order	1,27	5.53	0.026	1,27	0.10	0.759	1,27	0.93	0.342
Species	1,28	5.12	0.032	1,28	0.90	0.350	1,28	0.99	0.329
Stimulus	1,27	1.33	0.259	1,27	1.46	0.237	1,27	1.57	0.221
Species∗stimulus	1,27	4.52	0.043	1,27	0.09	0.771	1,27	0.71	0.407

The strength of aggressive response was expressed by three different response variables: PC1, PC2 and minimum distance. PC1 quantifies the increasing level of aggressiveness from approach with signs of interest to an attack on the taxidermic dummy during the experiment (see Table 1). PC2 quantifies the increasing amount of time devoted to singing during the experiment (see Table 1). Minimum distance is the minimum distance of the tested individual to the dummy during the experiment. The type of stimulus is represented by the predictor variable 'stimulus' (conspecific versus heterospecific), the order of stimuli by the predictor variable 'order' (first versus second) and species identity by the predictor variable 'species' (the common nightingale versus the thrush nightingale). The effects of particular predictor variables were estimated by linear mixed-effects models run separately for each response variable.

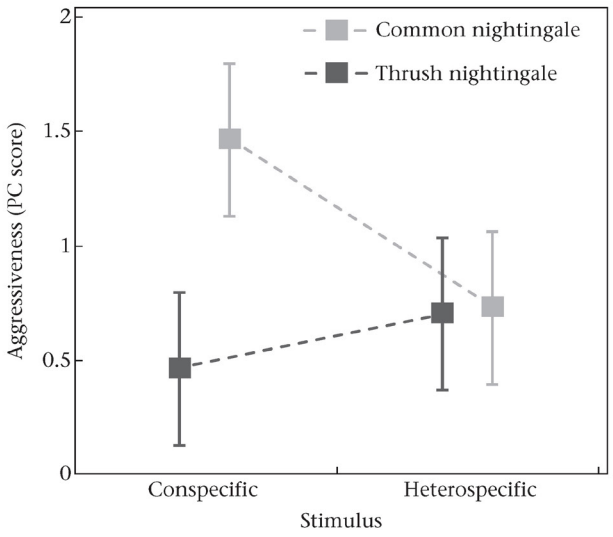


Figure 1. Aggressiveness of the behavioural response of nightingale males estimated by a linear mixed-effects model with the type stimulus (conspecific versus heterospecific) and species (the common nightingale versus the thrush nightingale) as predictors. Aggressiveness was quantified using scores along the first principal component (PC) axis revealed by PCA on six variables describing the behavioural response (see Table 1). Least-square means ± SE are depicted.

interspecific aggression is also consistent with the observation of interspecific territoriality on sites of co-occurrence (Ranoszek, 2001; Sorjonen, 1986) and implies the existence of interference competition between the two nightingale species in sympatry.

The previous study of Sorjonen (1986) suggested that males of the thrush nightingale, which is the larger and presumably competitively dominant species, are more aggressive in heterospecific reactions than common nightingale males. However, this finding could be a methodological artefact arising from the fact that stimuli were always presented in the same order (the thrush nightingale first, followed by the common nightingale) and the pause between stimuli was only 3 min in Sorjonen's study. Responses thus cannot be treated as independent; it seems likely that the reaction of tested thrush nightingale males to heterospecific stimuli was enhanced by the preceding conspecific playback (e.g. Lovell & Lein, 2004; Petrusková et al., 2008). We have not observed any such carryover effect in our study (which provided a sufficiently long, 1-day, break between the two stimuli). In fact, stronger reaction to the first playback stimuli resulted in a weak order effect, which was accounted for in the statistical analysis.

When we avoided the bias caused by the fixed order of stimuli in the playback experiments and quantified the level of males' response, we found that the smaller species, the common nightingale, was generally more aggressive. However, its higher aggressiveness was solely due to its stronger response to conspecific stimuli. When exposed to the heterospecific stimulus, the response of the common nightingale was much weaker, and did not differ significantly from that of the thrush nightingale. Interestingly, Stadie (1991) also suggested the common nightingale was more aggressive based on his observations of birds breeding in captivity. Our results suggest that competitive dominance of the larger thrush nightingale is not accompanied by increased aggressiveness. Similar examples have been described also in other avian and reptile species, although these typically involve distantly related taxa and not sister species (Martin & Ghalambor, 2014; Vanhooydonck, Van Damme, & Aerts, 2000).

A similar level of aggressiveness in the response of the thrush nightingale to both conspecific and heterospecific stimuli is unusual. Typically, territorial males react only to intrusions by conspecifics (Catchpole & Slater, 2008), and if heterospecific territoriality is observed, the reaction to conspecifics is usually stronger (e.g. Gil, 1997; Toms, 2013) as observed in the common nightingale. In the thrush nightingale, the asymmetry in interspecific copying of songs seems to be responsible for the observed pattern. Most males of the thrush nightingale in sympatry include parts of the common nightingale song in their repertoires, while such mixed singing does not occur in the common nightingale in sympatry (Becker, 2007; Vokurková et al., 2013) or is extremely rare (Lille, 1988). Furthermore, repertoires of some of these thrush nightingale mixed singers are actually dominated by the common nightingale song types (Vokurková et al., 2013). This copying could have an effect on the intensity of response of the thrush nightingale to the common nightingale song. Even a 'pure' common nightingale song recorded from allopatry presented in our experiment might be perceived by the focal thrush nightingale male as a song sung by a conspecific 'mixed singer'. In contrast, the common nightingale male would not mistake the heterospecific song presented in the experiment for the song of its own species, because we used recordings of the thrush nightingale from allopatry where the mixed singing was not detected (Vokurková et al., 2013).

Mixed singing in closely related bird species is usually considered to be maladaptive, resulting from erroneous learning (Helb et al., 1985). For example, in collared flycatchers, *Ficedula albicollis*, and pied flycatchers, *Ficedula hypoleuca*, mixed singing increases the rate of interspecific hybridization in sympatry and its frequency declines with the time since secondary contact (Haavie et al., 2004). As discussed in Vokurková et al. (2013), however, song convergence does not seem to substantially increase the rate of hybridization between nightingales. Vokurková et al. (2013) proposed two mechanisms by which the mixed singing in nightingales may have an adaptive value: (1) intraspecific female choice with preference for more complex songs resulting in increased fitness of the mixed singing thrush nightingale males in sympatry; and (2) territorial interactions between males of the two competing species. This latter alternative is supported by results from our playback experiments. We suggest that mixed singing in the thrush nightingale could be an adaptive response to interspecific competition. More similar heterospecific song may result in better competitor recognition and thus distinction of territory boundaries between the two species (Cody, 1969; Tobias et al., 2014). A similar relationship between song similarity and the intensity of male territorial response of co-occurring species has already been observed in *Hypocnemis* antbirds (Tobias & Seddon, 2009) and crested larks, *Galerida cristata*, and thekla larks, *Galerida theklae* (Laiolo, 2012). Together with our results, these studies show how interference competition may drive phenotypic convergence, which is a more common outcome of interspecific interactions than previously assumed (Tobias et al., 2014).

Further studies are needed, however, to provide more direct evidence on the possible role of song convergence in male–male aggressive interactions and its adaptive value in nightingales. We suggest that it can be tested by exposing individual males of both species to the sympatric songs with different levels of interspecific copying and/or different types of copied song. Furthermore, experiments investigating whether a tendency to mixed singing has a genetic basis should be performed (Grether et al., 2009).

Acknowledgments

The research was supported by grants provided by the Czech Science Foundation (15-10884Y) and the Grant Agency of the Charles University (632712). H. Kunc, R. Elwood and an anonymous referee provided very helpful comments on the manuscript.

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Appendix

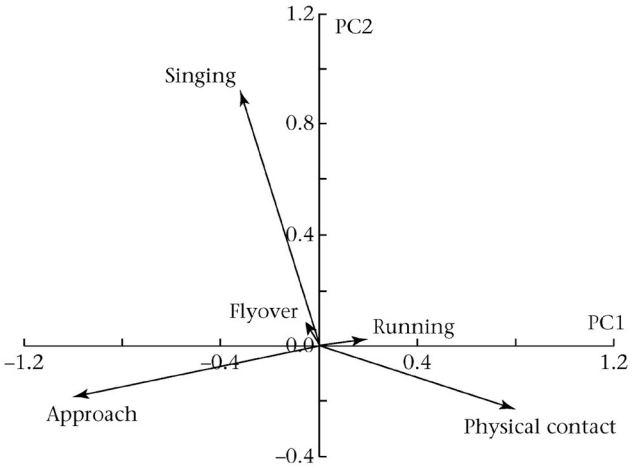


Figure A1. Relationships between particular types of behavioural response of males of the common nightingale and the thrush nightingale during playback experiments and first two principal component axes (PC1 and PC2) revealed by PCA. Note that the response type 'flight-attack' is not depicted owing to the absence of correlation with any of the axes.

Appendix 3

Souriau A, Geberzahn N, Ivanitskii VV, Marova IM, **Vokurková J**, Reifová R, Reif J, Petrusková T (2019) Singing behind the stage: thrush nightingales produce more variable songs on their wintering grounds. *Behavioral Ecology and Sociobiology* 73, 150.

Behavioral Ecology and Sociobiology (2019) 73: 150
https://doi.org/10.1007/s00265-019-2765-x

ORIGINAL ARTICLE

Singing behind the stage: thrush nightingales produce more variable songs on their wintering grounds

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Received: 5 May 2019 / Revised: 17 October 2019 / Accepted: 24 October 2019 / Published online: 19 November 2019
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Abstract

The songs of migratory passerine birds have a key role in mate attraction and territory defence during the breeding season. Many species also sing on their wintering grounds, but the function of this behaviour remains unclear. One possible explanation, proposed by the song improvement hypothesis, is that the birds take advantage of this period to develop their singing skills for the next breeding season. If so, non-breeding songs should reflect features of an early phase in song development, characterized by high vocal plasticity. In our study, we tested this prediction by comparing songs of thrush nightingales (*Luscinia luscinia*) recorded at two different breeding areas in Europe and one wintering area in Africa. While all songs from European localities had a typical structure characteristic of the study species, 89% of the songs recorded from Africa were highly variable, lacking such typical structure. We conducted further detailed analysis of breeding and winter songs that exhibited species-specific structure. First, we explored plasticity at the syllable level using a cross-correlation analysis, to obtain similarity scores as a measure of consistency. Second, we asked multiple human observers to quantify element variability. Our results showed significant differences in syllable consistency between breeding and wintering grounds, with more consistent delivery of syllables in the breeding areas. Likewise, element variability was substantially lower in the breeding populations. While both results fit the predictions of the song improvement hypothesis, more research is needed to elucidate the roles of singing on the wintering grounds.

Significance statement

Many migratory songbirds sing on their wintering grounds, outside the breeding period. While the role of singing during breeding has been broadly studied, our understanding of the function of winter singing remains limited. We analysed songs of the thrush nightingale, a migratory songbird with highly complex songs, comparing song structures recorded from breeding populations in Europe and an African wintering site. We demonstrate that males recorded at wintering locations sang songs with both significantly lower syllable consistency and higher element variability. Such characteristics are comparable to those observed during the sensorimotor phase of song development, previously described in other species. This pattern supports the song improvement

Abel Souriau and Nicole Geberzahn contributed equally to this work.

Communicated by J. Podos

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00265-019-2765-x) contains supplementary material, which is available to authorized users.

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hypothesis, suggesting that males singing on the wintering grounds may practice songs for the next breeding season. This study contributes to the understanding of the functions of songbird vocal behaviour out of the breeding context.

Keywords Thrush nightingale · Song function · Song plasticity · Song consistency · Element variability · Non-breeding singing

Introduction

Since Darwin published his book on selection in relation to sex (Darwin 1871), biologists have been intrigued by the functions of sexually selected characters. According to the sexual selection theory, two processes are important for such traits: male-male competition leading to armaments and female choice leading to ornaments (Berglund et al. 1996). Such traits have been intensively studied in the context of reproduction, but we have little knowledge about their functions outside of the breeding context. Birdsong is an example of such a sexually selected character. Males of most songbird species (oscines) that breed in the temperate zone sing intensively during the breeding period to repel rivals and to attract females. The singing performance of male individuals is thus a crucial component in estimating their quality for both competitors and potential partners (e.g. Catchpole and Slater 2008; Gil and Gahr 2002). While this behaviour has been well studied during the breeding period, why birds sing outside the breeding season remains an open question (Sorensen et al. 2016). Long-distance migrants spend a significant part of the year wintering at distant locations. Despite accumulating evidence of songbirds singing on their wintering grounds (e.g. Alexander 1917; Bannerman 1931; Cramp and Perrins 1994), research on this topic is still scarce.

A few studies investigating singing on the wintering grounds suggest that this behaviour may serve for territorial defence (e.g. marsh warbler, *Acrocephalus palustris*, Kelsey 1989), while others could not confirm such a territorial function (e.g. willow warbler, *Phylloscopus trochilus*, Sorensen 2014). Sorensen et al. (2016) tested several hypotheses related to the role of winter singing in great reed warblers (*A. arundinaceus*). First, they showed that seasonal variation in testosterone levels seems not to be responsible for triggering the singing behaviour. Second, no clear territorial behaviour was observed outside the breeding season. As an alternative explanation, the authors thus proposed the "song improvement hypothesis", suggesting that practicing complex songs during the non-breeding season could improve song performance for the following breeding season, and thus positively impact individual fitness.

Unlike many other sexually selected traits, the song of songbirds is a learned behaviour that is transmitted culturally (Catchpole and Slater 2008; Zeigler and Marler 2008). Young songbirds store model songs heard from conspecifics shortly after hatching during an early sensitive phase. They then gradually adjust their vocal output to what was stored before (Brainard and Doupe 2002). During this sensorimotor phase, the song is first characterized by high vocal plasticity ('plastic song'), and then gradually develops into more consistent songs (Brenowitz et al. 1997). Eventually, the song crystallizes and reaches its final adult and generally stereotyped form ('full song' or 'crystallized song'; Brainard and Doupe 2002). However, even after crystallization, the song of adult males may retain some plasticity. Such retention has been documented in adult males recapitulating plastic song later in life during an annual sensorimotor phase occurring during the non-breeding season (Nottebohm et al. 1986; Margoliash et al. 1991). The capacity to retain some plasticity may allow the birds to refine, improve and adjust their song motor performance even in adulthood (Sakata and Vehrencamp 2012).

Song improvement via practice thus seems to require adult birds to retain or temporarily regain some plasticity in their song. Consistent with this idea, Blyth's reed warblers (*A. dumetorum*) recorded during spring migration produced songs with less organized syntax and less stable syllable combinations compared to populations from breeding areas (Marova et al. 2010). Likewise, common nightingales (*Luscinia megarhynchos*) exhibited higher degrees of song variability in the wintering population compared to those at breeding localities (Kipper et al. 2016). However, neither of these studies investigated vocal plasticity by directly measuring syllable consistency. Syllable consistency is defined as the ability to replicate the acoustic features of a song from one rendition to the next (Sakata and Vehrencamp 2012). Consistent songs are expected to be adaptive since they send a more efficient signal with a higher-performance value, as high vocal consistency has repeatedly been linked to age, social dominance and reproductive success in several species (reviewed in Sakata and Vehrencamp 2012), and thus should be particularly important during the breeding season. Likewise, the plasticity of songs may be reflected in the variability of elements (the smallest units of a song), i.e. the acoustic structure of individual elements may vary from one rendition to the next. High variability of elements is thus evidence for low vocal consistency.

In our study, we compare the syllable consistency and element variability in thrush nightingales (*Luscinia luscinia*) recorded during the breeding and wintering period, respectively. We assume that if a song improvement process is at work, it should affect the consistency in the fine structure of the song resulting in less stereotyped songs. We explore the acoustic variation between renditions within each individual's performance (i.e. in one and the same recording), allowing us to

investigate the plasticity of song and the variability of its elements and compare if these individual song patterns differ between populations from breeding and wintering sites. Following the song improvement hypothesis, we predict that individuals recorded at the non-breeding site should display a higher level of vocal plasticity, and thus less stereotyped songs with a lower consistency in syllable performance than those from breeding grounds. Additionally, higher song plasticity might also result in higher element variability, as different reiterations (or rendition) of one element type might lack sufficient consistency in order to be perceived as the same type. To our knowledge, this is the first detailed quantitative analysis of wintering song based on spectral and temporal song characteristics. Such a structural approach may contribute to further understanding of the evolution and functions of non-breeding singing in migrating species.

Methods

Studied species and its song

The thrush nightingale is a long-distance migrating songbird with complex songs (e.g. repertoire varying from 23 to 42 songs, mean 32.7 per individual, Griessmann and Naguib 2002), characterized by a typical two-part ending phrase consisting of slow syllable repetitions, usually followed by a fast rattling part. This specific composition remains stable over the whole species range (Sorjonen 1983; Griessmann and Naguib 2002; Vokurková et al. 2013; Ivanitskii et al. 2016; cf. Fig. 1a). Closely related to the well-studied common nightingale, thrush nightingales seem to have the same ability to learn new songs even after their early life sensory phase and to adjust their song repertoire according to their neighbours (Sorjonen 1987; Griessmann and Naguib 2002). In secondary contact zones, thrush nightingales sometimes even learn from their sister species, the common nightingale (Vokurková et al. 2013). Learning after their early life sensory phase as well as learning from their sister species can have direct consequences on their vocal territorial interactions during the breeding season (Naguib and Todt 1998; Reif et al. 2015; Souriau et al. 2018). As a result, the mean proportion of song types shared between breeding males can be as high as 47% in a stable population (Ivanitskii et al. 2016). Moreover, syllable sharing seems to be even more pronounced: Griessmann and Naguib (2002) showed that birds from the same population shared up to 80% of their syllable types but only 30% of song types.

Every year, thrush nightingales migrate from Northern and Eastern Europe to East and South Africa (Pearson and Backhurst 1976), where they are known to sing actively (Pearson 1984), though their territorial habits and social interactions at their winter grounds remain poorly known. More recent studies investigating migration using geolocators suggest that birds from the same population may migrate to similar wintering regions (Stach et al. 2012; Thorup et al. 2017).

Also, males are highly philopatric to their breeding grounds (e.g. return rates up to 40% in Scandinavia, Sorjonen 1987), implying that the same individuals may continue to engage in vocal interactions in subsequent years.

Studied populations

This study is based on a comparison between three distant populations (i.e. males recorded in the same area) of thrush nightingales sampled over different years. All recordings presented "pure-species" songs, i.e. songs without any heterospecific components (elements from the closely related common nightingale, Vokurková et al. 2013). Populations from breeding grounds in Europe were recorded in Poland, Podlasie (53°12.0′N, 22°24.4′E), during spring 2009, and in Russia, Zvenigorod, Moscow region (55°42.3′ N, 36°44.6′E), during spring 2012 and 2013. The wintering individuals were recorded in Tanzania, Mbeya region (8°47.7′ S, 34°03.0′E), from January to March 2006, over 6000 km away. The distance between the two breeding localities is 970 km. This ensures a minimal connectivity between the breeding populations and provides different pools of syllables and elements for our analysis, as song repertoire varies with distance in thrush nightingales (Sorjonen 1987).

Populations from the breeding areas were typically found along river banks or woody vegetation patch edges in close neighbouring territories, with a maximum distance of 5 km between sampling sites within a given population. Such males apparently belong to the same sub-population, and thus share a relatively large part of their syllable repertoire, which remains stable and identifiable over several years (AS, unpublished data from Polish and Russian localities). In contrast, the African recordings were made along a 20 km gradient, likely sampling a wider diversity in song repertoire due to the longer distance between individuals, and possibly also their different origin. Although only one population from the wintering ground was analysed in detail due to sample size and recording duration requirements, we also considered several available recordings from Kenya, Malawi, Zambia, Botswana and South Africa (JV unpubl. data, Macaulay Library of Natural Sounds (http://macaulaylibrary.org), Xeno-canto database (http://www.xeno-canto.org)). The use of two well-separated breeding populations, and examination of recordings from other wintering sites, allowed us to reduce the risk that a simple population effect causes the observed differences in syllable consistency and element variability between breeding and wintering grounds. Although we cannot fully rule out such population effects, our approach focussing on syllable consistency within individual repertoires should eliminate the direct effects of geographic variation.

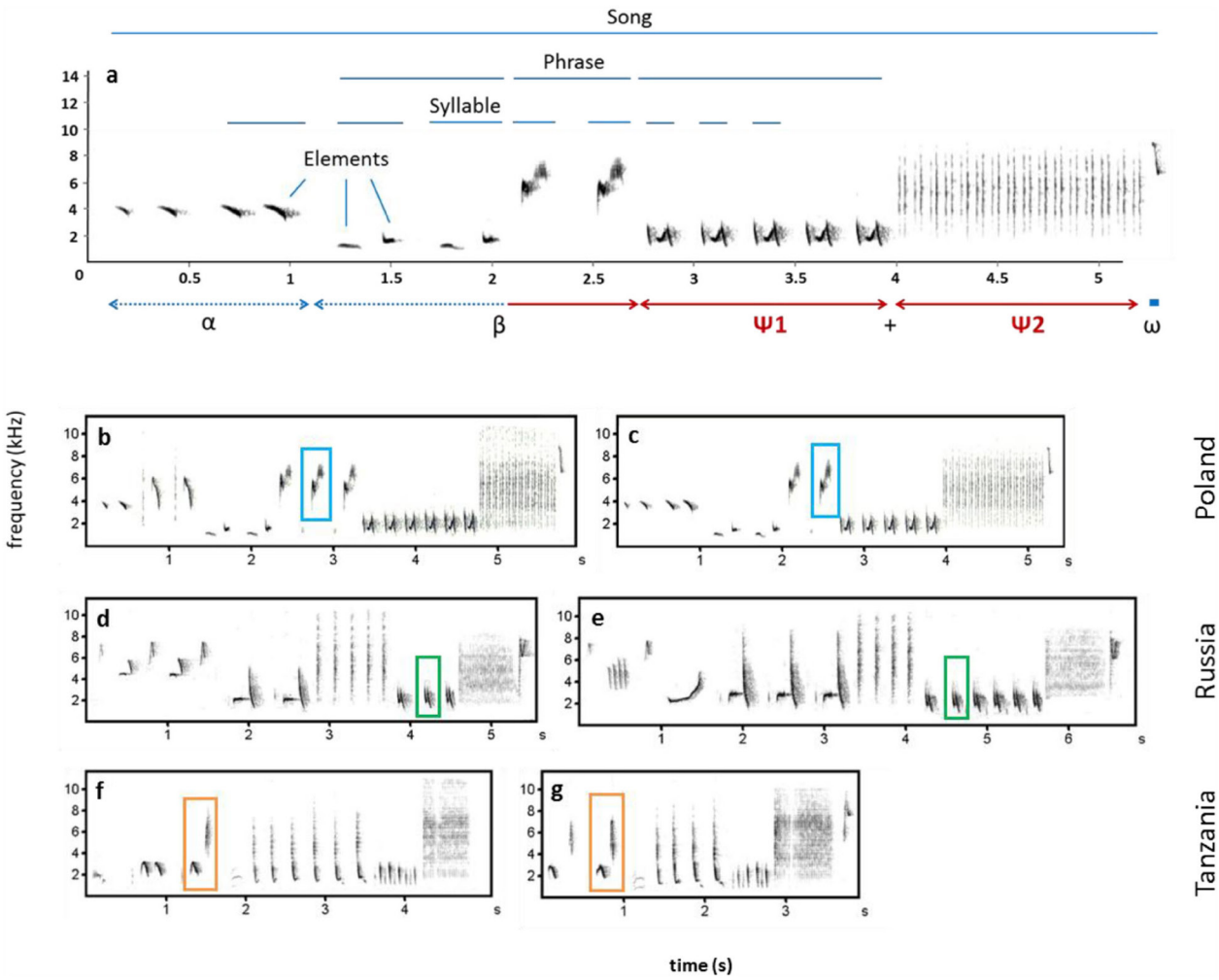


Fig. 1 **a** Spectrogram of a typical thrush nightingale song (recorded in Poland) showing the characteristic song organisation of the species. The sub-parts (indicated by Greek characters and highlighting the ending "castanet" and "rattling" parts as introduced by Sorjonen 1983) are characterized by elements that always occur in the same sequence. The α-part is usually the most variable part between and within individual repertoires, while the last parts of the song (solid red arrow lines) are the most stable ones. In this study, song type categories are defined as the same combination of the last β element and the following Ψ1 (Castanet) and Ψ2 (Rattling, fast trill covering a wide frequency range), indicated by solid arrow lines underneath the spectrogram. **b**–**g** Spectrograms illustrating syllable renditions selected for the spectral cross-correlation analysis and their sequential position: **b**, **c** Poland; **d**, **e** Russia; **f**, **g** Tanzania. Highlighted syllables illustrate renditions selected for comparison of the same syllable type in two songs of the same type and from the same individual

Data collection in the field

Recordings used for this study were made during the morning (0400–1300 h, Russia and Tanzania) or both the morning and evening (0600–0900 and 1900–2000 h, Poland). In the majority of cases, we recorded spontaneously singing individuals. For some recordings from Poland, we used the playback of conspecific songs to elicit vocal behaviour; the potential effect of such playbacks has been controlled for in the analysis (see below).

Our non-invasive recording approach did not involve any marking of individuals. Therefore, particular care was given to the song recording strategies and subsequent selection of recordings to rule out the risk of repeated analysis of the same individual's song. Only one recording was used per any given recording location, representing confirmed territorial birds in the two breeding populations. At the wintering locality, the birds were usually recorded at greater distances from each other than at the breeding sites, thus minimizing the possibility that one male was recorded twice. Another limitation of studying unmarked birds is the lack of information on the age or pairing status of the subjects. Thus, while individuals of the breeding populations can be considered adults aged 1 year or older, at least some individuals on the wintering ground could have been in their first year (we come back to this issue in the discussion).

Recordings were generally collected from a cautious distance to minimize disturbance of the bird's natural behaviour. The mean overall duration (mean duration ±SD) of recordings was Poland = 20.3 ± 2.2 min (n = 15), Russia = 15.6 ± 2.2 min (n = 12), Tanzania = 14.0 ± 4.0 min (n = 7). Recording was performed using Sennheiser ME67 (Poland) or ME66 (Russia, Tanzania) directional microphones, connected to Marantz PMD660 (Poland, Russia) or PMD670 (Tanzania) solid-state digital recorders. It was not possible to record data blind because our study involved focal animals in the field.

Song analyses

Song definition

In this study, we define a syllable type as the smallest invariant unit composed of one or a few elements (i.e. the smallest iteration in the spectrogram) that are always repeated in the same sequential order (Fig. 1a). Syllables are usually organized into larger consistent units called "songs", typically separated from each other by silent pauses (see examples of three different song types in Fig. 1b–g). Songs of thrush nightingales are generally characterized by the presence of two subsequent ending phrases, referred to as "castanet" and "rattling" by Sorjonen (1983). Given the higher variability of syllables at the beginning of the song, even within the same individual, the definition of a song type for this study is based on a combination of the typical two-part ending and the last phrase preceding it (Fig. 1a) traditionally used for descriptions of thrush nightingale song (Sorjonen 1987).

The songs selected for our quantitative analyses had to pass a two-step evaluation: first, they had to exhibit clearly identifiable thrush nightingale species-specific song organization and characteristic ending phrases (see Fig. 1a), as in previous studies from different breeding sites (Sorjonen 1983, 1987; Naguib and Todt 1998; Marova et al. 2015a; Ivanitskii et al. 2016) (Fig. 2). Songs and/or recordings that were lacking the typical syntax and repetition patterns (similar to 'sub-songs') were not considered further. Second, those selected in the first step (either typical songs of the species or variants presenting some degrees of variation in their syntax and sequence of elements, similar to 'plastic songs') had to successfully pass an automatic detection procedure necessary to ensure the accurate assignment of syllable types (see below).

Three syllable categories were defined to cover different aspects of their structural diversity and position in the song, and thus potential associated constraints in vocal production. We aimed to test three syllable types for each individual, one for each category (see Fig. 3, see the full list of tested syllables in Online Resource 1):

- Category A: disyllabic association of two discrete syllables or elements that are always performed one after the other. Those types are usually found among the first stable elements of the recurring sequence defining a song type (see last part of β syllables in red, Fig. 1a).
- Category B: this category comprises complex syllables characterized by highly repeated structures, long duration and/or harmonics at either low or high frequency. Similar lower pitched "buzz" structures have been suggested to be costly in the common nightingale (Weiss et al. 2012), due to potential physiological constraints of high-speed production. In thrush nightingales, complex syllables are usually found in the β parts of the songs (see Fig. 1a), except for the Russian population in which they are used as final syllables.
- Category C: low pitched "castanet-like" syllables with narrow frequency bandwidth that are highly repeated in the song. They constitute a mandatory part of the characteristic ending of thrush nightingale songs (Sorjonen 1983, 1987).

Populations in Poland and Russia exhibited local syllable sharing between individuals (see Online Resource 1), allowing the selection of comparable syllable types from songs of different individuals for the analysis. In contrast, the absence of such syllable sharing between individuals in Tanzania led to the selection of a larger diversity of syllable types. However, our measure of syllable consistency is not impacted by this difference in the degree of syllable sharing between different populations, as syllables were compared within songs of single individuals.

Syllable consistency

We measured individual syllable consistency by conducting a semi-automatic intra-individual comparison between several occurrences of the same syllable type across different songs. We thus calculated the average spectral cross-correlation (SPCC) of a given syllable rendition in comparison to another rendition of the same syllable type selected from another song of the same type at an analogous serial position (see Fig. 1b–g). For each individual, in the same recording, one syllable rendition was chosen as a reference pattern for each of the three syllable categories, whereby priority was given to renditions with high signal to noise ratios and without masking sounds.

The cross-correlation analysis was performed with Avisoft-SASlab Pro 5.2 (www.avisoft.com, Specht 2007) as follows: first, the syllable repertoire of birds was visually explored to assess the quality of potential candidate syllables. Based on this exploration, appropriate syllable types were preselected according to the criteria of recording quality and whether there was a sufficient number of recurrences at analogous serial positions inside the same song type. The selection of renditions on analogous serial positions was necessary to rule out any potential effects of the serial position (see Fig.

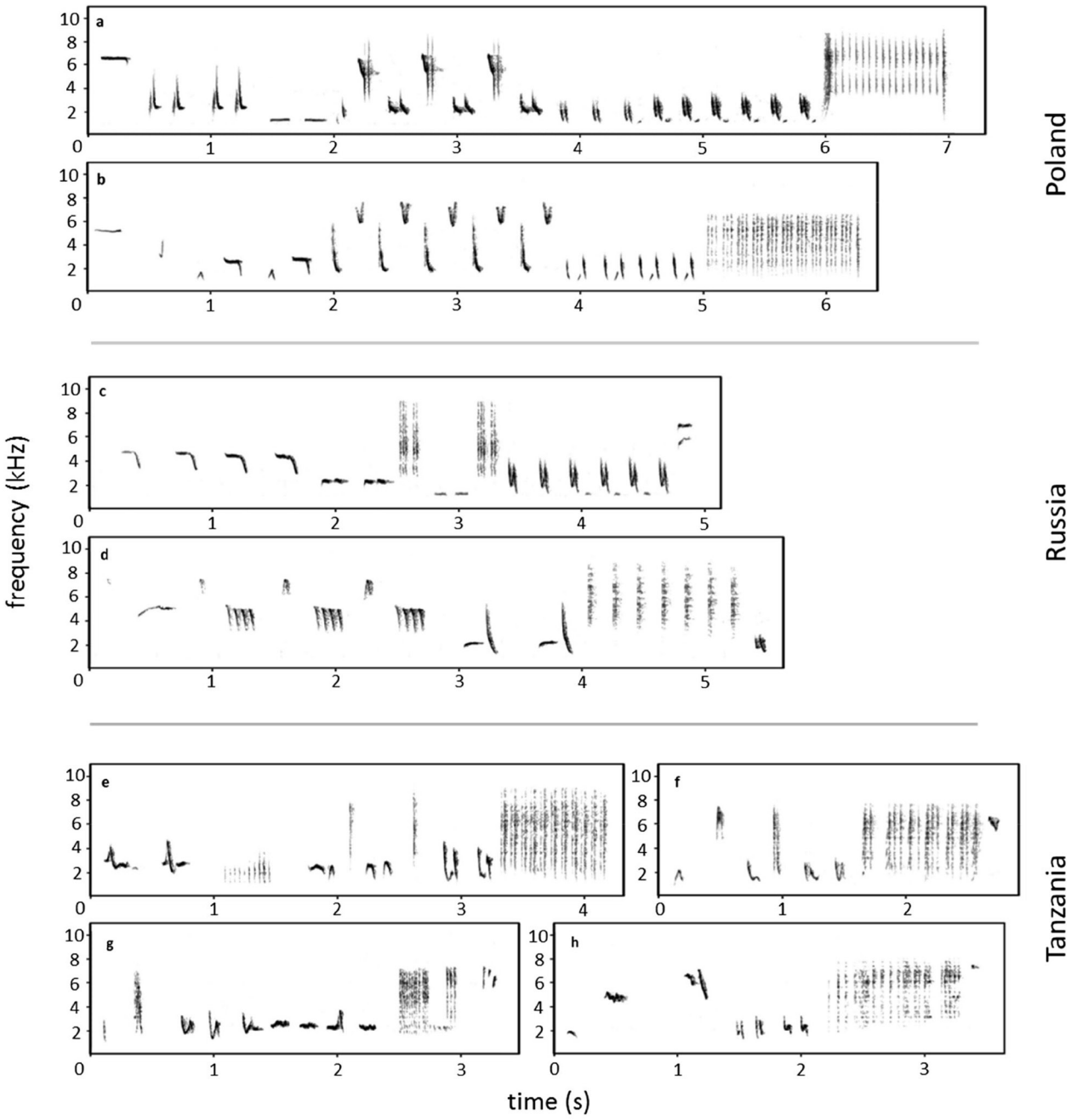


Fig. 2 Spectrograms of songs from each population. Note the clear organisation of the thrush nightingale song in **a**, **b** Poland and **c**, **d** Russia, contrasting with the variability in syllable shapes and repetition patterns visible in various elements of the Tanzanian examples. Song **e** is representative of the sample successfully used in the cross-correlation analysis, while songs f–h display an increasing level of syllable plasticity, typical of the recordings excluded due to the absence of clear repeatable song patterns

1b–g). Next, we applied the "scan for template spectrogram patterns" function in Avisoft-SASlab for automatic recognition of syllable types. To this end, we used the frequency band cut-off function to compute only those frequency bands covered by the given syllable type, minimizing the effects of potential background noises. Settings were set to exclude any frequency deviation (0 Hz) or masking interval (0 s). To improve this automatic procedure, the recognition of syllables was visually confirmed (false positive = wrong syllable detected) and guided (false negative = matching syllable undetected at the selected threshold) by one human observer (AS). The same observer also carried out the preselection and was

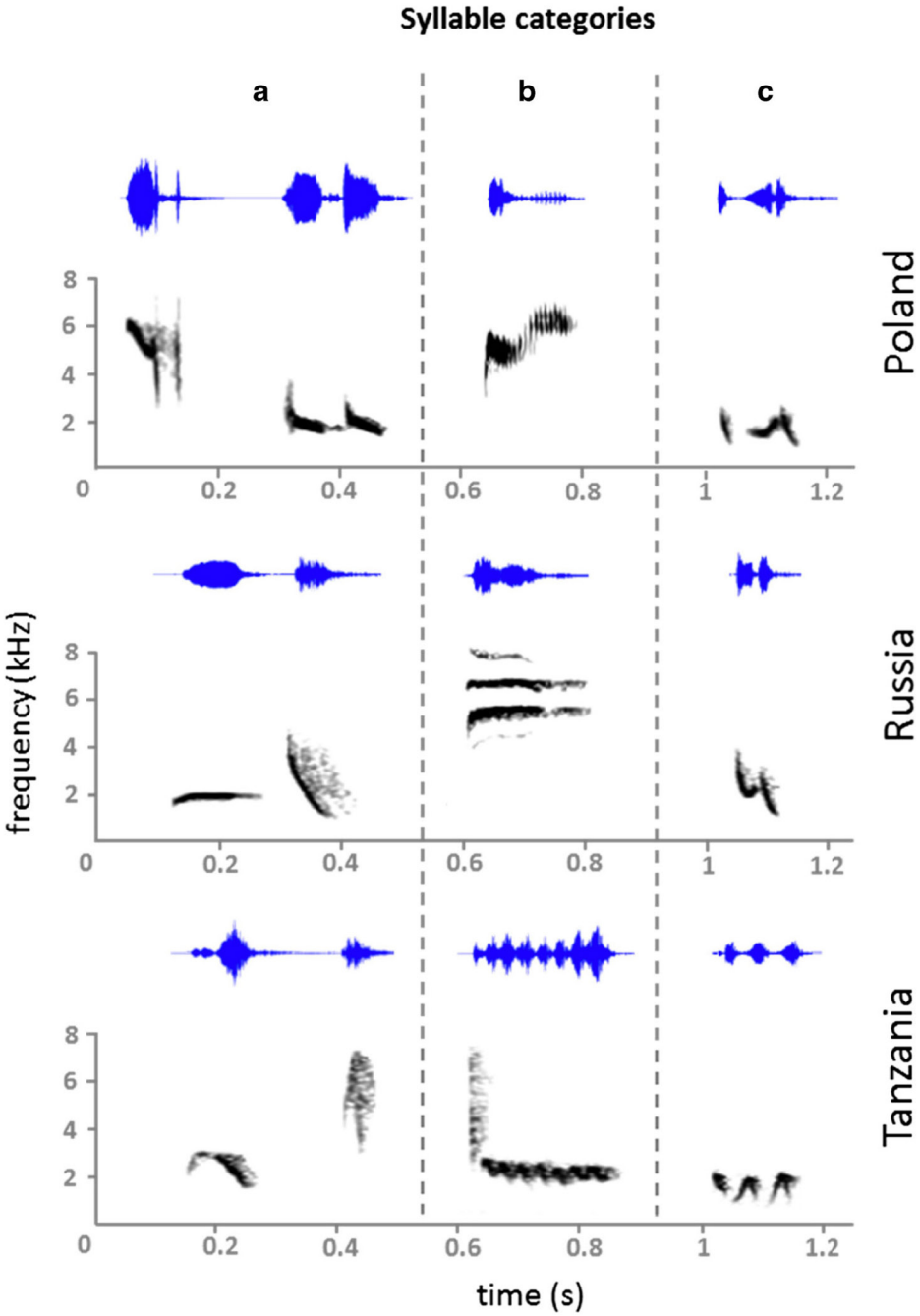


Fig. 3 Spectrograms (bottom) and oscillograms (top) illustrating examples of selected syllable types used for the cross-correlation analysis in the three different categories (A = disyllable association, B = complex syllables, C = low pitched "castanet") for each locality

not blind as to the population origin of the recordings. Given this semi-automatic approach, the risk of a potential observer-expectancy bias (e.g. Brumm et al. 2017) is expected to be low.

For the recording of each individual that passed the two-step selection procedure, we analysed three syllable types, each representing one of the three categories previously introduced (see examples in Fig. 3), except for one Tanzanian individual for which we could not find an appropriate syllable type for category A and two other Tanzanian individuals with no appropriate syllable types for category B. The mean number (±SD) of renditions of the same syllable type in the same individual compared per category was, for each population: Poland A = 21.1 ± 5.7, B = 11.2 ± 3.8, C = 10.5 ± 2.2; Russia A = 18.8 ± 6.5, B = 12.3 ± 5.5, C = 10.8 ± 4.8; Tanzania A = 9.43 ± 4.1, B = 10.20 ± 2.5, C = 10.67 ± 3.5.

Element variability

We performed an additional analysis to further investigate song characteristics at the element level. Due to the subjectivity of defining limits for an element category when songs presented continuous variability, we used ten different human observers. The observers, experienced with spectrograms of birdsong but not of thrush nightingales and blind to the population origin of the given individuals or tested hypotheses (to avoid any potential bias), were provided with the same set of spectrograms. Each of our three populations was represented by five individuals selected from the same sample as used for the analysis of syllable consistency, and each individual by five songs (resulting in a total of 75 songs). The observers were then instructed to visually assess the number of element types as well as the total number of countable elements present in each song. Given the structural specificity of highly repeated long trills in the typical songs of our species and the technical difficulty in counting the number of elements in the fastest ones, all the broadband clicks repeated more than 3 times in a row (e.g. final trills in Fig. 1) were scored as one "serial element".

Element variability within each evaluated song was expressed as the ratio of different element types to the total number of elements per song (equivalent to "song versatility" in Järvi 1983; Gil and Slater 2000; Gil et al. 2001). Lastly, a mean index value for an individual bird was calculated based on each observer's mean score from the five songs of a given individual. We expected element variability to be reflected in the spectrograms by a larger number of different element types sung.

Statistical analysis

Our hypothesis that birds from the wintering grounds sing less stereotyped songs with a lower consistency in syllable performance was tested by a comparison of (1) the syllables' SPCC for each of our three syllable categories and (2) the element variability index between our three populations with a focus on the contrast between breeding and non-breeding grounds.

We used LMMs (linear mixed-effect models) implemented in R software 3.2.2 (R Core Team 2016). To test for a global difference in the syllable correlation scores between populations, we first fitted full models (nlme package, Pinheiro et al. 2017) with the syllable correlation score as a dependent variable, with the presence or absence of playback used in order to elicit song and population origin as fixed-effects predictor variables and bird's identity as a random factor. The models were then corrected by either including or not including the potential playback effect based on the output of the previous models for each category separately.

A similar approach was used to test for an overall difference in element variability, with the element variability index as a dependent variable, the population origin as a fixed-effects predictor variable and the bird's identity as a random factor. A potential influence of playback on the element variability index was not tested by the model, as all the individuals from Poland used for this analysis except one were stimulated, and such an effect would then be confounded into the Polish population category.

To correct for multiple testing, we adjusted the α values of global tests using the sequential Bonferroni procedure. For LMMs that revealed a significant effect of any predictor variables, we used post hoc Tukey's contrasts for multiple comparisons (multcomp package, Bretz et al. 2010), which provided P values adjusted by the single-step method.

Ethical note

Samples used in this study are based on a non-invasive recording technique from a distance. Birds were not caught, but for few individuals, playback stimulation was performed to trigger an active singing behaviour. This stimulation was kept as short as possible and we are not aware of any consequences for subjects' breeding or welfare. Authorizations for fieldwork were granted from legal authorities in each different sampling country when needed. For the wintering population, recordings were made within the framework of another research project (Geberzahn et al. 2009, 2010).

Results

While all songs in the recordings from both breeding populations had the typical structure of thrush nightingale song, this was far from being the case in the wintering population: when pooling all recordings across different recording locations, 59% of all Tanzanian recordings (39 out of 66) did not pass the first step of the selection process, as their songs were highly disorganised (i.e. most or all of the typical sub-parts α, β, $\Psi 1$, $\Psi 2$ and ω could not be identified, cf. Fig. 1a) and/or missed silent pauses in between. The recordings available from other known wintering grounds (Kenya, Malawi, Zambia, Botswana and South Africa) showed the same patterns of plastic and disorganized song structure as described above for Tanzanian recordings.

Recordings that did not pass the first step of the selection process could not be considered further as our analysis relied on the ability to assign syllables to syllable types. In contrast, we did not have to exclude any recordings from Poland and Russia (Fig. 2). Of the remaining 27 recordings from Tanzania, only 7 successfully passed the automatic detection procedure (each of them corresponding to a different recording location). Thus, in total, 89% of the Tanzanian recordings (59 out of 66) did not enter the subsequent analysis due to extremely low structural consistency, while all recordings from the breeding sites (15 from Poland and 12 from

Russia) passed both steps and were suitable for the quantitative analysis of intra-individual syllable consistency.

Intra-individual syllable consistency

Our measure for syllable consistency (SPCC) showed a significant overall difference between populations for all three categories (Table 1a). In all of them, birds from Tanzania displayed significantly less consistent syllables than both Polish and Russian ones (Table 2, Fig. 4).

Significant differences were also found between breeding populations in categories A and B. While birds from the Russian population produced more consistent category A syllables than birds from the Polish population (Table 2, Fig. 4a), the patterns were reversed for the category B syllables, sung less consistently by Russian birds than by Polish ones (Table 2, Fig. 4b).

Element variability

The analysis based on the element variability index obtained by assessments of ten human observers showed a significant difference in element variability between populations (Table 3a). As predicted, songs produced by males wintering in Tanzania showed a higher element variability index than songs from both Polish and Russian populations (Table 3b, Fig. 5). This clearly indicates that syllables in songs from the wintering population were more variable than those from breeding grounds, between which no significant differences in element variability were observed (Table 3b, Fig. 5).

Table 1 Overall contrast in syllable consistency scores (SPCC) between all three populations. (a) Comparisons from the modelling of the effect of population origin (as well as playback stimulation for categories B and C) on the syllable consistency score, for each syllable category (A = disyllabic associations, B = complex syllables, C = castanet-like syllables). (b) Results from the preliminary modelling of the effect of both the playback stimulation and population effect on the syllable consistency score. Note that for the category A, the factor playback was removed from the final model)

Syllable category (a)	numDF	denDF	*F*	*P* (population effect)
Category A	2	30	18.025	< 0.001
Category B	2	28	17.149	< 0.001
Category C	2	30	28.031	< 0.001
Syllable category (b)	**numDF**	**denDF**	***F***	***P* (playback effect)**
Category A	1	29	1.697	0.203
Category B	1	28	12.339	0.002
Category C	1	30	28.031	0.031

Discussion

Thrush nightingales recorded on their wintering grounds in Tanzania sang with significantly lower consistency than individuals recorded at two different breeding locations. Our measure of syllable consistency was lower for wintering birds in all three selected syllable categories. Likewise, wintering birds performed songs with more variable elements than did birds from the two breeding populations. Our multiple-population approach allowed us to reduce the potential influence of a simple population effect as the two breeding populations displayed similar patterns, both differing from the wintering one, and additional recordings from other sites in Africa were comparable to those from Tanzania. Below, we consider our findings in light of the song improvement hypothesis proposed by Sorensen et al. (2016). Furthermore, we discuss other potential causes for singing on the wintering ground, based on both field and developmental studies.

Higher vocal plasticity in the wintering population

Songs performed by thrush nightingales on their Tanzanian wintering ground exhibited a strikingly lower syllable consistency and higher element variability than the songs produced by individuals of two different populations from breeding grounds. Concerning the lower syllable consistency quantified by cross-correlation analyses our results are highly conservative, given that more than half of the Tanzanian recordings did not pass the first step of the selection, and about 3/4 of the remaining songs had to be excluded in the second step because of the high variability between different syllable renditions, making assignments to the same syllable type impossible. Therefore, taken as a whole, songs of thrush nightingales were substantially less consistent in Tanzania than estimated by our quantitative analysis.

Such vocal plasticity is usually associated with early subsong (soft, rambling sounds) and plastic song produced during the sensorimotor phase of the song learning process, in contrast to the more consistent song produced during the breeding season (e.g. Marler and Peters 1982; Hultsch and Todt 2004; Catchpole and Slater 2008). Although we cannot rule out that our Tanzanian sample contains songs of first-year males, by excluding recordings from our analysis that showed structures highly reminiscent of developmental sub-song and plastic song, our data should rather represent birds past their first year of life. Therefore, such vocal plasticity likely reflects older males engaging in a later plastic phase (Margoliash et al. 1991), although studies with marked birds of known age would be necessary to confirm this hypothesis. In any case, we consider that the lower syllable consistency in the wintering population revealed by our analysis supports the song improvement hypothesis, as defined in Sorensen et al. (2016). Higher element variability in the wintering population

Table 2 Contrast in syllable consistency scores (SPCC) between pairs of populations. The table shows all pairwise comparisons from the modelling of the effect of population origin (as well as playback stimulation for categories B and C) on the syllable consistency score, for each syllable category (A = disyllabic associations, B = complex syllables, C = castanet-like syllables)

Syllable category	Populations	Estimate	SE	*Z*	*P*
Category A	Poland–Tanzania	0.152	0.037	4.089	< 0.001
	Russia–Tanzania	0.230	0.038	5.998	< 0.001
	Russia–Poland	0.008	0.029	2.760	0.016
Category B	Poland–Tanzania	0.235	0.040	5.856	< 0.001
	Russia–Tanzania	0.145	0.038	3.854	< 0.001
	Russia–Poland	− 0.090	0.032	− 2.82	0.013
Category C	Poland–Tanzania	0.195	0.030	6.433	< 0.001
	Russia–Tanzania	0.193	0.028	6.876	< 0.001
	Russia–Poland	− 0.002	0.027	− 0.092	0.995

also likely results from higher vocal plasticity in winter singing. The more variable performance of syllables might have hindered human observers in their assigning of aberrant syllable renditions to the corresponding syllable type. To some extent, however, higher element variability in the wintering populations could be indeed caused by males producing a larger number of different syllable types per song.

While the consistency for all three syllable type categories was always lowest in recordings from Tanzania, the differences between Russian and Polish population were not consistent (Fig. 4). Additionally, the results for the category C (castanet-like syllables, characterized by long repeated sequences and simple structure) suggest an overall higher consistency compared to the categories A and B within each population. This pattern might be explained by a potential learning facilitation for syllables of category C, which are usually exhibited in long repetitive phrases found across the species range (see Sorjonen 1987).

Singing in wintering birds

Our results show that thrush nightingales predominantly produce plastic song when singing on their wintering ground in Tanzania. Similar contrasts have been observed between recordings of thrush nightingales from other known breeding grounds (e.g. Sorjonen 1983; Griessmann and Naguib 2002; Vokurková et al. 2013; Ivanitskii et al. 2016) and from other wintering grounds, including Kenya, Malawi, Zambia, Botswana and South Africa, which display songs with a lack of element consistency comparable to the one described in our analysis (AS personal observation, Online Resource 3). This

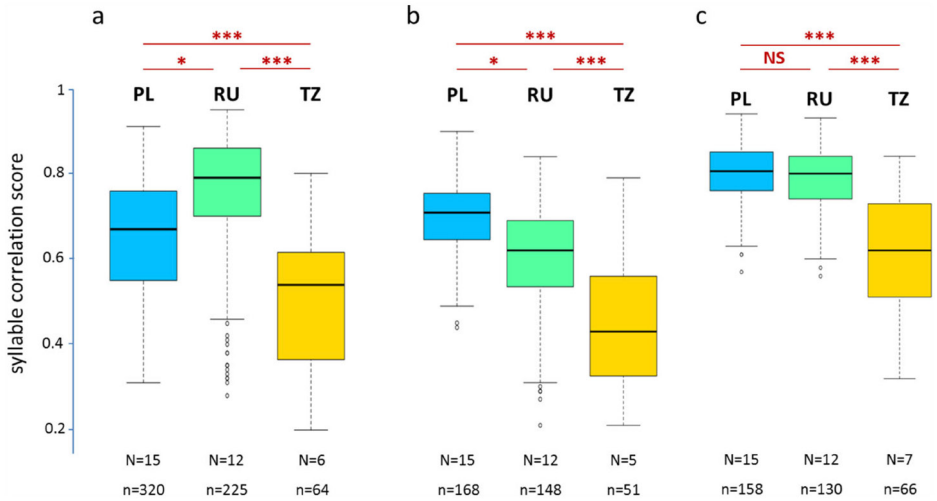


Fig. 4 Syllable correlation score (SPCC) as a measure of syllable consistency in three populations of thrush nightingales. Shown are three syllable categories: **a** disyllabic associations (category A), **b** complex syllables (category B), and **c** castanet-like syllables (category C). From left to right: Poland (PL), Russia (RU), Tanzania (TZ). Box-and-whisker plots depict the median, interquartile range and non-outlier range (whisker length up to 1.5 times the interquartile range). Numbers underneath the plots represent the total number of observations (*n*) and the number of subjects (*N*). Note that the individual identity of birds was entered in the corresponding LMM as "random = (~ 1|ID)" in order to avoid pseudoreplication. Post hoc Tukey tests from the linear models were used to conduct pairwise comparisons of populations for each syllable category (NS = $p > 0.05$, *$p < 0.05$, ***$p < 0.001$)

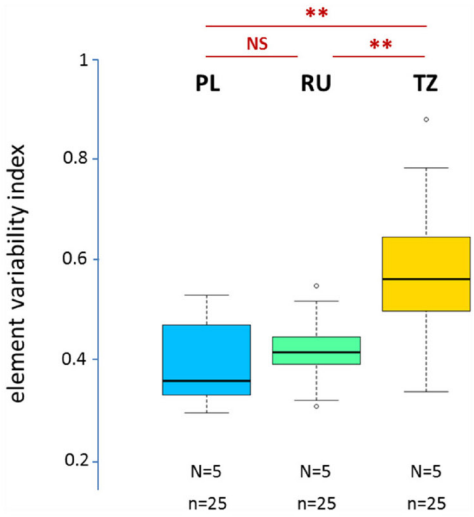


Fig. 5 Element variability index in three populations of thrush nightingales assessed at the element level. From left to right: Poland (PL), Russia (RU), Tanzania (TZ). Box-and-whisker plots depict the median, interquartile range and non-outlier range. Numbers underneath the plots represent the total number of songs (*n*) and the number of individuals (*N*) tested. Note that the individual identity of birds was entered in the corresponding LMM as "random = (~ 1|ID)" in order to avoid pseudoreplication. Post hoc Tukey tests from the linear model were used to conduct pairwise comparisons of populations for each syllable category (NS = $p > 0.05$, **$p < 0.01$)

suggests that plastic song on the wintering grounds is widespread at the species level rather than a rare behaviour. Moreover, our findings are concordant with the results of a study on the common nightingale singing at its wintering grounds in Gambia (Kipper et al. 2016). While their analysis did not assess song consistency quantitatively, the authors noted that the element accuracy and stereotypy of elements seemed lower for most recordings from the non-breeding period. Comparable results were also found in the great reed warbler (*Acrocephalus arundinaceus*), another species with high song complexity and a similar migration strategy. Great reed warblers were found to use longer songs and switched more often between different syllable types at their winter grounds (Sorensen et al. 2016). In the Palearctic region, Blyth reed warbler (*Acrocephalus dumetorum*) individuals from the most southern areas of their distribution, most likely migrating individuals, were also found to sing songs with "chaotic syntax and instable phonetic structure" (Marova et al. 2010; Samotskaya et al. 2016). To support and extend these examples, a recent comparative meta-analysis from Sorensen et al. (2016) found a significant link between the occurrence of song in migratory bird species wintering in Africa, and the complexity of their produced song. We may speculate that the occurrence of a singing phase on wintering grounds is widespread, and actually essential, for those species in which the development of complex song extends beyond an early sensory phase of song learning on their breeding grounds (Table 3).

Table 3 Contrast in element variability index between populations. (a) The overall contrast in the element variability index between all three populations. The results of all pairwise comparisons from the modelling of the effect of population origin for each syllable category (A = disyllabic associations, B = complex syllables, C = castanet-like syllables)

(a)

	numDF	denDF	*F*	*P*
Population effect	2	30	18.025	< 0.001
(b)				
Populations	Estimate	SE	*Z*	*P*
Poland–Tanzania	0.173	0.049	3.491	0.001
Russia–Tanzania	0.148	0.049	2.980	0.008
Russia–Poland	0.025	0.049	0.510	0.866

Proximate aspects of plasticity in wintering song

The occurrence of plastic song in wintering birds may be related to the fact that the acquisition of new song characteristics correlates with seasonal changes of the song control regions in the brain over consecutive years, as has been shown in some species (e.g. Nottebohm 1981; Tramontin and Brenowitz 2000). However, whether thrush nightingales reduce their song control nuclei seasonally is currently unknown. In other species that do not exhibit such pronounced seasonal changes, it has been shown that the variability in song structure seems to be actively generated by the song system, as lesions in certain song nuclei can cause a reduction of song variability (e.g. Brainard and Doupe 2000; Kao and Brainard 2006). Song plasticity may thus be functional and allow continuous modification of the song through rehearsal and adjustment to the social environment even in adulthood (e.g. Sakata et al. 2008; Sakata and Vehrencamp 2012; Woolley and Doupe 2008; Tumer and Brainard 2007).

Complementary insights from developmental studies

Song ontogeny has not been directly investigated under controlled laboratory conditions in thrush nightingales. However, in the closely related common nightingale, young males kept in the laboratory spend several months singing plastic song during the winter and early spring (thus whilst their wild conspecifics are on the wintering ground) and crystallize their song at the beginning of the first breeding season (Hultsch and Todt 2004). Furthermore, captive common nightingales go through additional phases of plastic singing before their song crystallizes again in subsequent breeding seasons (Henrike Hultsch, personal communication). This additional phase of plastic singing seems to be a prerequisite for males to integrate song types into their motor program that they have

newly acquired during their first season (Todt and Geberzahn 2003), highlighting the potential role of plastic singing as a form of motor exploration in adulthood. Moreover, in common nightingales, hand-reared yearlings not only delayed the use of some early tutored songs to the 2nd year of life but also produced imitations more often in their 2nd year if the same tutored songs were broadcasted again when the individuals were about 1 year old (Geberzahn et al. 2002; Geberzahn and Hultsch 2003). In that regard, singing during the winter season could have a role in activating pre-existent memory as well as increasing and adjusting a bird's song repertoire to its social environment.

Such adjustments might be particularly important in species that use vocal matching to address each other in vocal territorial disputes, such as common nightingales (Todt and Naguib 2000) or song sparrows (*Melospiza melodia*, Beecher 2008). In that context, sharing parts of one's repertoire with territorial neighbours may convey advantages, and such vocal sharing has been reported in many species (see Handley and Nelson 2005 for review). This change in repertoire would thus allow better song matching and conflict regulation between neighbours of the same population over years in highly philopatric species. Thrush nightingales seem to be able to add new elements to their song repertoire over successive breeding seasons, as documented by Sorjonen (1987) and suggested by Griessmann and Naguib (2002). Thus, singing during the wintering period could not only benefit young males developing their singing skills, but also older males adjusting their repertoire to new songs heard during the previous breeding seasons.

Complementary and alternative hypotheses for wintering song

Our results are in accordance with the "market place" hypothesis (Kipper et al. 2016), which proposes that singing outside of the breeding season could be an opportunity for older birds to learn and share new songs with nearby wintering individuals. Wintering grounds would then constitute a song "market place" where males would go through a new sensory phase of song learning, allowing better territorial communication with neighbouring conspecifics over time. From this point of view, the "market place" hypothesis can be regarded as a part of the song improvement hypothesis.

Regardless of the potential delayed benefits for a migrating bird to sing outside of its breeding season, further investigations will be needed to substantiate that wintering song serves as song improvement. Likewise, we cannot rule out other possible functions. The strong territorial behaviour of thrush nightingale males during the breeding season does not seem to be widespread on the wintering grounds, and particular individuals singing highly variable songs could sometimes be observed singing quite close to each other (NG personal observation). Nonetheless, a competition over feeding resources might still occur. In order to determine whether winter singing serves a territorial function in thrush nightingales, future playback studies in Africa would be needed to test whether variable songs recorded from the wintering grounds are potent enough to elicit a territorial response.

Conclusion

This study reveals conspicuous differences in song performance between breeding and wintering grounds in thrush nightingales. Our results improve the understanding of how a culturally transmitted sexually selected trait like birdsong, playing a central role in reproductive success, might be influenced by a complex learning process with functional implications over different seasons. Future studies should aim to track individual birds over their full life cycle in order to provide direct evidence for song improvement. Additionally, further investigations exploring the strategies of non-breeding singing behaviour and its possible functions are needed to achieve a clear understanding of songbirds' singing behaviour throughout their full life cycle.

Acknowledgments We want to thank all the colleagues involved in the assessment of element variability for their precious help: Ondřej Belfín, Paolo Biella, Sharina van Boheemen, Hana Kahounová, Jan Mertens, Alexandra Průchová, Lucie Jiránková and especially Adam Petrusek for his overall support and help with the manuscript. NG would like to thank Wolfgang Goymann, Sonja and Andreas Kümmerle and Liz and Neil Baker for logistic support as well as Mackubi Joseph for help with fieldwork in Tanzania. VI and IM are grateful to Vladislav Antipov for the recording of nightingales in Russia. We also thank David Hardekopf for language corrections. Additional thanks go to Tony Archer, James Bradley, Josh Engel, Niall Perrins and Derek Solomon for kindly consenting to the use of their recordings. Lastly, we are grateful to two anonymous reviewers and Jeff Podos for their valuable comments on a previous version of the manuscript.

Funding This work was supported by the Association for the Studies of Animal Behaviour (ASAB) to NG; the Deutsche Forschungsgemeinschaft (grant numbers GE 1253/3-1 and GE 1253/3-2 to NG); the Russian Fund for Basic Research (grant number 16-04-01721 to VI) and the Russian Science Foundation (grant number 14-50-00029 to VI).

Compliance with ethical standards

Ethical approval All applicable international, national, and institutional guidelines for research on animals were followed. Samples used in this study are based on a non-invasive recording technique from a distance. Birds were not caught, but for few individuals, playback stimulation was performed to trigger an active singing behaviour. This stimulation was kept as short as possible and we are not aware of any consequences for subjects' breeding or welfare. Passive recording did not require

supervision from institutional or regulatory bodies, but authorizations were granted from legal authorities in each different sampling country when needed. In the wintering population, recordings were made within the framework of another research project (for more details see Geberzahn et al. 2009, 2010).

Conflict of interest The authors declare that they have no conflict of interest.

Data accessibility Analyses reported in this article can be reproduced using the data provided in Souriau et al. 2019 (data Tables with recordings information and spectrograms prints used for the human-based assessment of element variability).

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