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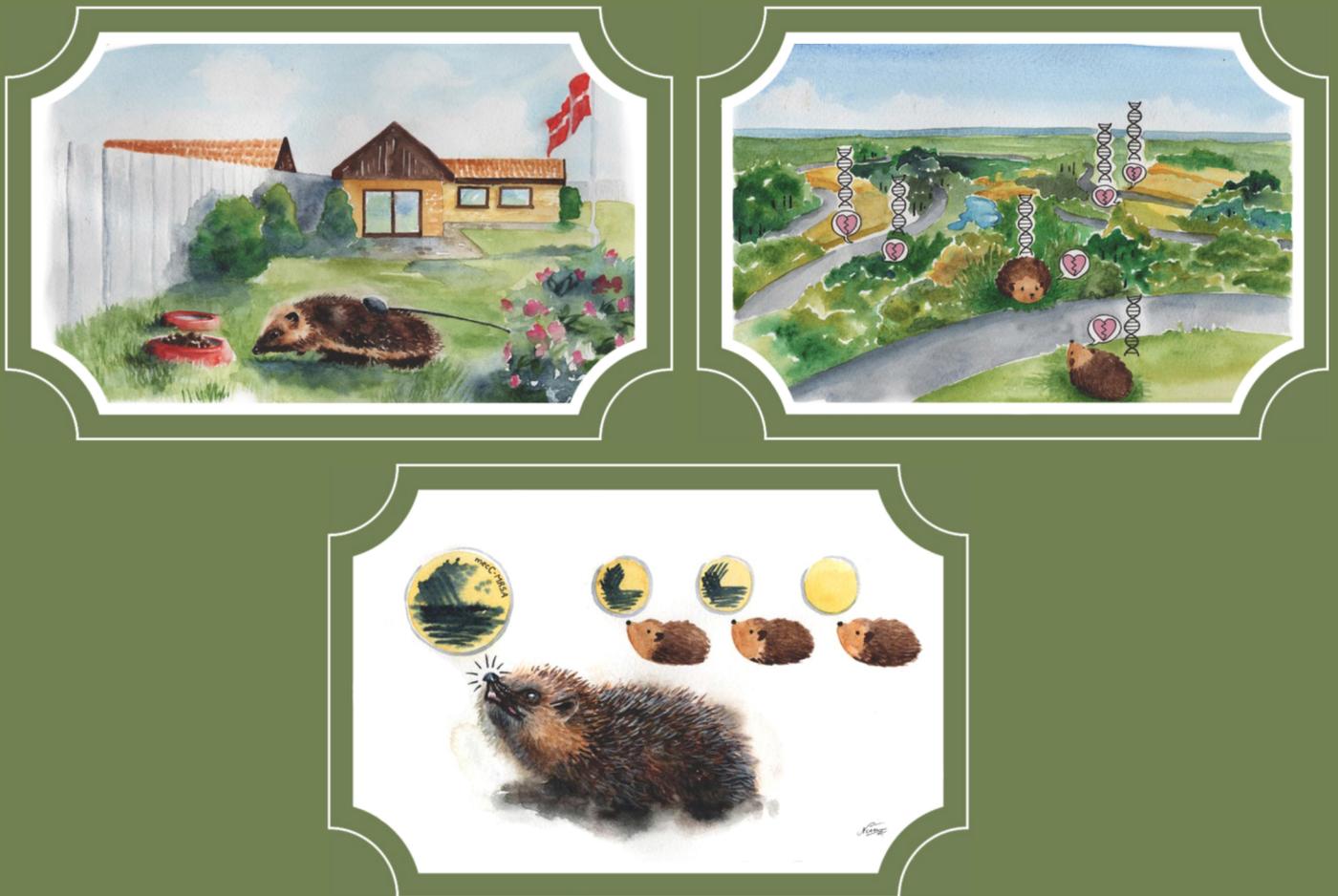


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THE DANISH HEDGEHOG PROJECT

HOW HUMANS INFLUENCE HEDGEHOGS AND
HOW HEDGEHOGS MAY AFFECT HUMANS WHEN
LIVING CLOSELY TOGETHER

PHD THESIS
SOPHIE LUND RASMUSSEN



SUPERVISORS:
OWEN R. JONES AND THOMAS B. BERG

DEPARTMENT OF BIOLOGY
UNIVERSITY OF SOUTHERN DENMARK

JUNE 2019

SDU 

The Danish Hedgehog Project

How Humans Influence Hedgehogs and How Hedgehogs May
Affect Humans When Living Closely Together

*Thesis submitted in partial fulfillment of the requirements for the
degree of Doctor of Philosophy (PhD)*

by

Sophie Lund Rasmussen

Supervisors:

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NATURAMA

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Preface

This thesis is a product of my PhD project entitled The Danish Hedgehog Project, which I initiated in 2016 with the generous combined support of the University of Southern Denmark and Naturama. The initial idea was to use dead hedgehogs to understand the living, by gathering types of knowledge regarding the general Danish hedgehog population's health, which is otherwise problematic to achieve on living hedgehogs, due to ethical concerns and protection restrictions. Thus, the project engaged Danish citizens through citizen science to collect dead hedgehogs from every corner of Denmark to form the basis for my research. My optimistic hope was to receive 300 dead hedgehogs from the period of May-December 2016, but the massive media attention I managed to create for the project with over 200 newspaper articles, radio interviews and TV features in the national and local news resulted in an unprecedented sample size of 697 dead hedgehogs, collected mainly by volunteers throughout Denmark. The unparalleled sample size furthermore represents the hedgehog population of a whole country! Danes are generally very fond of hedgehogs and people actively engaged in the project, connecting with the notion that the hedgehogs would not have died in vain when their remains were utilized to improve our understanding of Danish hedgehogs and hence optimize the conservation efforts to protect them.

Through necropsies of the 697 hedgehogs a vast range of samples were obtained. We, me and my team of research assistants, retrieved skin samples for genetics from the completely flat individuals consisting only of skin, along with information about the location and date of find, and where possible also information on the sex of the individual. Furthermore, we extracted 23 samples from the intact individuals and recorded the location, date of the find and sex of the individual, if the genitals were still present. It was a privilege to receive such a massive amount of dead hedgehogs. However, the extent of the workload was more than doubled compared to the planned timeframe of the project, which postponed the completion of the necropsies extensively and hence, delayed the research and analyses of the extracted samples. I prioritised to include all 697 hedgehogs in the project, as I considered it a waste of effort and opportunity to reduce the sample size to the originally planned 300 hedgehogs.

I established collaborations with a number of Danish specialists to ensure that the research produced would be of the highest possible quality. At the present time, we have the results of the genetic, MRSA, parasitological, dental health and age determination studies. In the years to come, my goal will be to finish the remaining planned research on gut microbiomes,

toxicology, morphometrics, diet choice, population modelling, *Salmonella* detection and histopathology of the hedgehogs. Furthermore, we have obtained a number of organs (kidneys and spleens from 219 individuals) and tissue samples, which have not yet been allocated to a particular course of study. Ultimately, when combining all this knowledge, I will be able to make conclusions on the conservation status of the Danish hedgehog population.

After two years of planning and applying for funding, it was a dream come true when my PhD project on hedgehogs became a reality. To my knowledge, it is the first PhD project ever to be conducted on hedgehogs in Denmark. The project was originally formulated and designed by me, and with the help of my supervisors, we managed to secure funding for two years of PhD salary and tuition fees. The normal framework of a PhD at the Department of Biology at the University of Southern Denmark is a three-year duration, where the student is obligated to obtain 30 ECTS points of PhD courses, teach for 840 hours (240 confrontation hours), carry out 300 hours of knowledge dissemination and complete a research stay at a foreign research institution for two to six months. Since it was impossible to find the funding for the last year of my PhD, I was fortunate to be granted a one year merit for my fieldwork on juvenile hedgehogs in suburban areas, which constitutes one of the manuscripts of this dissertation. Having only two years to complete a PhD has been a challenge, especially due to my own high expectations and the massive extent of the project. My obligations, apart from doing the actual research, have been to obtain 30 ECTS points of PhD courses, teach for 560 hours out of which 160 were confrontation hours, conduct 300 hours of knowledge dissemination and complete a research stay at Statens Serum Institut (SSI) for three months. All within the framework of two years. Moreover, the project was delayed for a year because of happy circumstances, when I became a mother in 2017. Regrettably, the limited timeframe of two years only allowed me to include three manuscripts in the present dissertation, even though more results were ready for publication. However, the inclusion of three manuscripts is also the required standard for the normal PhD projects, with three-year durations, at the Department of Biology, University of Southern Denmark.

The Danish Hedgehog Project has engaged and communicated with hundreds of volunteers collecting dead hedgehogs, 26 volunteers housing the collection stations throughout Denmark, and 12 volunteers assisting in the lab performing necropsies, cleaning and preparing the bones for inclusion in the museum collection at the Natural History Museum in Copenhagen, and conducting age determinations and dental health examinations. Furthermore, the project has involved approximately 50 research staff members ranging from research assistants to lab technicians, as well as professors, which is quite exceptional for a single PhD project.

I am very proud of the results obtained so far, and look forward to continue my research on hedgehogs, hoping that it will have an impact on both a political level and on a conservation level, preserving hedgehogs in the wild and ensuring that future generations will be able to experience this intriguing and characteristic mammal in its natural habitat.

I sincerely hope you will enjoy reading my thesis.

Sophie Lund Rasmussen

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I wish to express my heartfelt gratitude and appreciation to my PhD supervisors Owen Jones and Thomas Bjørneboe Berg for guiding me patiently and providing valuable advice, encouragement and for sharing their indispensable experience with me, which ultimately led to the successful completion of this thesis. Thank you for showing support and understanding for the several particular circumstances that affected the project. I am especially thankful to my supervisor Thomas Bjørneboe Berg, who went to great lengths to ensure the establishment of the PhD project and the majority of funding for my PhD salary and tuition fees, after I originally approached him with my research ideas and ambition to conduct a PhD on hedgehogs in Denmark.

I would like to thank my collaborators and their research staff for our inspiring, pleasant and rewarding collaborations:

- Torben Dabelsteen, Section for Ecology and Evolution, Institute of Biology, University of Copenhagen
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- Heidi Huus Petersen and Jakob Hallig, National Veterinary Institute, Technical University of Denmark

I am indebted to the Natural History Museum of Denmark and staff members Daniel Klingenberg Johansson, Eline Lorenzen and Mikkel Høegh Post for providing the facilities, practical help and specialist advice necessary for the necropsies and maceration.

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I would like to convey my gratitude to the following institutions and their staff, as well as individuals who housed and managed collection stations for dead hedgehogs in the period of May-December 2016:

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This accomplishment would never have been possible without the support of my family and friends, who have patiently listened to my countless tirades about the obstacles and challenges of the project, helping me to navigate through it practically as well as mentally. Furthermore, they have selflessly accepted to be lectured about hedgehog ecology even in the most inappropriate situations. Finally, I must express my deepest gratitude to my family, especially my mother Elisabeth Lund, mother-in-law Birgitte Pank Arbøll, sister Mathilde Kæreby Lund and my most beloved husband Troels Pank Arbøll for providing me with unfailing support and continuous encouragement throughout my years of study. Thank you for always believing in me and for wholeheartedly engaging in my research no matter how nerdy and intrusive it has been.

List of Manuscripts

- I. Sophie Lund Rasmussen, Thomas B. Berg, Torben Dabelsteen and Owen R. Jones,
The ecology of suburban juvenile European hedgehogs (*Erinaceus europaeus*) in a
changing Danish climate.

Submitted to Ecology and Evolution.

- II. Sophie Lund Rasmussen, Jeppe Lund Nielsen, Owen R. Jones, Thomas B. Berg and
Cino Pertoldi,

Genetic structure of the European hedgehog (*Erinaceus europaeus*) in Denmark.

Submitted to PLOS ONE.

- III. Sophie Lund Rasmussen, Jesper Larsen, Rien E. van Wijk, Owen R. Jones, Thomas
B. Berg, Øystein Angen and Anders Rhod Larsen,

European hedgehogs (*Erinaceus europaeus*) as a natural reservoir of methicillin-
resistant *Staphylococcus aureus* carrying *mecC* in Denmark.

Submitted to PLOS ONE.

Abstract

Accumulating research evidence suggests that the European hedgehog (*Erinaceus europaeus*) is in decline in several western European countries. It is therefore essential to understand the reasons behind this decline to optimise and direct conservation initiatives to protect the species in the wild. This thesis attempts to bridge the gaps in our knowledge of the survival challenges for hedgehogs residing in suburban areas, as well as the potential negative influence humans and hedgehogs may exert on each other, when sharing habitats.

In the first manuscript we described the survival and behavioural ecology of 35 radio-tagged juvenile hedgehogs living in suburban areas, during their first year of life, from September 2014-July 2015. We discovered that the juvenile hedgehogs had small home ranges and high survival rates compared to previous studies, and obtained a satisfying weight gain during the autumn. We furthermore observed that the mild climatic conditions during the autumn of 2014 affected the behaviour of the hedgehogs, as they remained active for a longer period of time than previously recorded. However, we also detected how anthropogenic effects such as feeding stations in gardens, could lead to the spread of lethal *Salmonella* infections between the hedgehogs, and how garden tools and loose dogs unfortunately caused deaths to a couple of individuals.

In the second manuscript we investigated the genetic composition of the Danish hedgehog population by adapting the GBS technique to the application of 178 hedgehog samples. The Danish population of hedgehogs was divided into three genetic clusters, representing a total of six different populations. The genetic variability in the Danish hedgehog population was low compared to results from other European countries. This tendency is likely generated by anthropogenic effects such as habitat fragmentation caused by human mediated structures, such as roads, creating isolated populations with a low effective population size.

The third manuscript focuses on the detection of methicillin-resistant *Staphylococcus aureus* (MRSA) carrying the *mecC*-gene in hedgehogs. We discovered that hedgehogs appear to be natural reservoirs of *mecC*-MRSA with a prevalence of 61% positive carriers out of 188 tested, and found no evidence for transmission to hedgehog rehabilitators, in spite of one previously reported case of *mecC*-MRSA transmission from a hedgehog to a human in Denmark.

The insight gained from the present research indicates that humans do affect the health and survival of the hedgehogs in Denmark, and that there is a potential risk of zoonotic transmission between hedgehog and humans, when sharing habitats and living closely together.

Dansk Resumé

Forskningsbaseret evidens indikerer, at det europæiske pindsvin (*Erinaceus europaeus*) er i tilbagegang i flere vesteuropæiske lande. Det er derfor af yderste nødvendighed at få beskrevet og etableret en forståelse for de bagvedliggende årsager til denne tilbagegang, for derved at muliggøre og optimere initiativer til at beskytte og bevare arten i naturen.

Målet med denne afhandling er at afdække de udfordringer pindsvinene møder i forstæderne, samt hvilken indflydelse mennesker og pindsvin kan udøve på hinanden, når de deler habitat og lever side om side.

Det første manuskript beskriver pindsvineungers overlevelse og adfærd i forstadshaverne på Københavns vestegn. Ved hjælp af radiosendere fulgte vi 35 selvstændige pindsvineunger i deres første leveår. Vi opdagede at ungerne havde små home ranges og høj overlevelse sammenlignet med tidligere studier, samt at størstedelen af ungerne tog godt på i vægt i løbet af efteråret. Vi observerede også at de milde lokale vejrforhold i efteråret 2014 påvirkede pindsvinenes adfærd, da de forblev aktive meget senere på efteråret end hidtil beskrevet. Vi så desværre også hvordan menneskeskabte påvirkninger, såsom foderstationer i haverne, blev en kilde til smitte med dødelige *Salmonella*-infektioner mellem pindsvinene, og at løse hunde og haveredskaber desværre kostede flere af pindsvineungerne livet.

Det andet manuskript omhandler den danske pindsvinebestands genetiske sammensætning. Vi tilpassede den såkaldte GBS teknik til brug på prøver fra 178 pindsvin, og kunne konkludere, at de danske pindsvin kunne inddeles i tre genetiske klynger, som i alt repræsenterede seks forskellige populationer. Pindsvinenes genetiske variation var temmelig lav sammenlignet med resultater fra andre europæiske lande. Den lave genetiske variation er sandsynligvis forårsaget af menneskeskabte faktorer som landskabsfragmentering, som eksempelvis det omfattende vejnet, hvor små, lokale pindsvinebestande bliver isoleret.

Det tredje manuskript fokuserer på fundet af methicillin-resistent *Staphylococcus aureus* bærende *mecC*-genet (MRSA) i pindsvin. Vores forskning viste, at pindsvin tilsyneladende er et naturligt reservoir for *mecC*-MRSA, da 61% af de testede 188 individer bar *mecC*-MRSA. Vi forsøgte at kvantificere risikoen for smitteoverførsel ved at teste pindsvineplejere fra Dyrenes Beskyttelse for MRSA, og ingen var smittet, selvom der tidligere er registreret ét enkeltstående tilfælde af *mecC*-MRSA smitteoverførsel mellem pindsvin og et menneske.

Vores forskning viser, at mennesker påvirker pindsvins sundhedstilstand og overlevelse i Danmark, og at der er en potentiel risiko for smittespredning mellem pindsvin og mennesker, når vi deler habitater og lever så tæt sammen.

List of Abbreviations

MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
N_e	Effective population size
H_o	Observed heterozygosity
H_E	Expected heterozygosity
uH_E	Unbiased expected heterozygosity
CDC	The Centers for Disease Control and Prevention
WHO	The World Health Organization
MLST	Multilocus sequence typing
DNA	Deoxyribonucleic acid
ST	Sequence types
CC	Clonal complexes
PBPs	Penicillin binding proteins
SCC mec	Staphylococcal Cassette Chromosome <i>mec</i>
LA-MRSA	Livestock-associated MRSA
DANMAP	Danish Integrated Antimicrobial Resistance Monitoring and Research Programme
GBS	Genotyping by sequencing
SNPs	Single-nucleotide polymorphisms
HWE	Hardy-Weinberg equilibrium
IBD	Isolation by distance
JNL	Jutland north of the Limfjord and Jutland south of the Limfjord
JSL	Jutland south of the Limfjord
F	Funen
Z	Zealand
LFA	Lolland Falster
BH	Bornholm
<i>spa</i>	Staphylococcal protein A
F	Inbreeding coefficients
P%	Percentage of polymorphic loci
BWA	Burrows-Wheeler Aligner
MAF	Minor allele frequency
F_{ST}	Fixation index
K	Genetically distinct populations/groups
PCA	Principal component analysis
IAM	Infinite allele model
ANOVA	Analysis of variance
CMR	Capture-mark-recapture
KDE	Kernel density estimation
MCP	Minimum convex polygons
GLM	Generalized linear model
AIC	Akaike information criterion
BI	Bunnell Index
DMI	Danish Meteorological Institute
PBS	Phosphate-buffered saline
MIC	Minimum inhibitory concentration
EUCAST	The European Committee on Antimicrobial Susceptibility Testing
CLSI	Clinical and Laboratory Standards Institute
CORINE	Coordination of Information on the Environment
PCR	Polymerase chain reaction
SAK	Immune evasion molecules staphylokinase (<i>sak</i>)
CHIPS	Chemotaxis inhibitory protein of <i>Staphylococcus aureus</i> (<i>chp</i>)
SCIN	Staphylococcal complement inhibitor (<i>scn</i>)

Introduction

Previous research indicates that the European hedgehog is declining in western Europe. It is therefore essential to gather knowledge on hedgehog ecology in order to understand what is causing this decline, and thereby direct and optimise conservation efforts leading to the preservation of the species in the wild. The aim of this thesis is to investigate how humans influence hedgehogs when sharing habitats and living closely together, and how hedgehogs may affect humans through potential zoonotic transfer of infections.

The scientific approach of the thesis was the completion of three different case studies. The first sought to describe the ecology of juvenile hedgehogs residing in suburban areas with attention on the anthropogenic effects on hedgehog survival and health, in order to fill out knowledge gaps enabling us to improve the conservation initiatives directed at preserving hedgehogs in suburban and urban areas (Manuscript I). We also conducted a nationwide study on the genetic diversity of hedgehogs to determine the degree of inbreeding among the Danish hedgehog population, and thereby improve our understanding of their conservation status (Manuscript II). In this study, we furthermore attempted to ascertain the anthropogenic factors accountable for the low heterozygosity found. Lastly, to explore the potential influence hedgehogs may exert on humans, we studied the prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) carrying *mecC* in a nationwide sample of Danish hedgehogs. *mecC*-MRSA has a zoonotic potential, especially when hedgehog and humans often come into direct or indirect contact in residential gardens and at hedgehog rehabilitation centres. To quantify this risk, we subsequently tested a number of hedgehog rehabilitators for *mecC*-MRSA (Manuscript III).

The samples for the research on genetics and MRSA came from a nationwide Danish collection of dead hedgehogs based on citizen science, which was initiated as part of the PhD project. A total of 697 dead hedgehogs were collected from all over Denmark in 2016. The three case studies of this thesis provide new knowledge on hedgehogs which may individually contribute to the improvement of future conservation efforts directed at preserving the species in the wild.

This chapter serves to provide the background knowledge necessary for interpreting the results in the three manuscripts, which constitutes this thesis.

The European hedgehog (*Erinaceus europaeus*)

The western European hedgehog (*Erinaceus europaeus*) is a spiny mammal species found on the British Isles, Continental Europe from Iberia and Italy northwards into Scandinavia, as well as on New Zealand. It is a generally common and widely distributed species that can survive across a wide range of habitat types (Morris 2018; Reeve 1994). Hedgehogs are nocturnal and non-territorial, and they primarily rely on their well-developed hearing and sense of smell when foraging at night (Morris 2018). They feed on a wide range of prey, ranging from macroinvertebrates to other prey types such as amphibians, birds and their eggs, small mammals, carrion and human food found in the environment (Wroot 1984; Yalden 1976). Hedgehogs spend up to 70 % of their active time foraging (Riber 2006).

Nowadays, hedgehogs increasingly inhabit areas with human activity, for instance parks in urban areas, gardens in residential areas and rural villages (Hubert et al. 2011; Pettett et al. 2017; van de Poel et al. 2015). As a consequence, many garden owners generously supply food and water for their local hedgehogs (Morris 2014), thereby making hedgehogs habituated to human presence. The hedgehog is therefore one of the few wild mammals people are prone to come into physical contact with. Hedgehogs can travel up to 2-3 km per night, and the home ranges of adult hedgehogs are generally estimated to be 20-30 ha for males and 10 ha for females, expanding temporarily during the mating season (Morris 2014). Juvenile hedgehogs do not disperse far from their natal area when they reach independence (Sæther 1997), and adult hedgehogs seem to remain in the same area throughout their lives (Reeve 1994). Furthermore, relocated individuals do not disperse very far (Rasmussen 2013), even when released into an unfavourable habitat (Doncaster et al. 2001).

Hedgehogs go into hibernation as a strategy to overcome the challenge of finding enough food to accommodate their metabolic costs during the cold months, where most of their preferred food items are not available (Morris 2018). In Scandinavia, previous research has established that the onset of hibernation is normally between October and November, depending on the sex and age of the individuals, and activity is resumed from mid-April to mid-May (Jensen 2004; Kristiansson 1984; Kristiansson 1990; Rasmussen 2013; Walhovd 1976; Walhovd 1978; Walhovd 1990). The same studies also showed, that hedgehogs change nests 0-4 times during winter and the winter survival rate is between 60-100 %.

Hedgehogs are seasonal breeders and their sexual behaviour commences soon after the termination of hibernation, which is dependent on weather conditions, although it usually takes place from mid-April to mid-May in Denmark (Walhovd 1976). For male hedgehogs, the testis

activity starts in January while the animal is still hibernating. Spermatogenesis and testosterone secretion peak in May after the male's arousal from hibernation (Reeve 1994). Female hedgehogs show a succession of oestrous cycles and are spontaneous ovulators (Morris 2018). Hedgehogs are promiscuous and have heteropaternal superfecundation (Moran et al. 2009). Their courtship behaviour is noisy and aggressive, and males compete for the favour of the females (Morris 2018). Females can give birth to two litters in one season, but it has only recently been reported in Denmark (Manuscript I). Walhovd (1984) found numerous examples of young born in August-September in Denmark. Few litters seem to be born before late July in Denmark. This is supported by the fact that the Danish Animal Welfare Society (Dyrenes Beskyttelse) does not receive orphaned hedgehogs for rehabilitation until late July. Pregnancy lasts for approximately 34 days, and the litter size in Denmark is normally 4-6 individuals, with an average of 4.7 per litter (Walhovd 1984). In Sweden, Kristiansson (1984) estimated an average litter size to be 5.2 and the British average is 4-5 (Morris 2018).

Hedgehog decline

Previous research on both national and local scale has either documented decline or indicated concerns for the decline of hedgehog populations in several western European countries (Hof & Bright 2016; Krange 2015; Müller 2018; SoBH 2011; SoBH 2015; SoBH 2018; van de Poel et al. 2015; Williams et al. 2018). During the past decades, a range of different approaches have been used to monitor the hedgehog population in the UK (Morris 2018). But unlike the UK, there is currently no data to indicate the status of the Danish population of hedgehogs. Nonetheless, the situation may be similar in Denmark due to the comparable habitat fragmentation, landscape structure, farm management practices and climate in the western European countries. The suspected reasons for this decline include habitat loss and habitat fragmentation, intensified agricultural practices, roadkills, molluscicide and rodenticide poisoning and badger predation (Brakes & Smith 2005; Dowding et al. 2010a; Dowding et al. 2010b; Haigh et al. 2012; Hof & Bright 2010; Hubert et al. 2011; Huijser & Bergers 2000; Pettett et al. 2017; SoBH 2011; Young et al. 2006)

Hedgehog research in Denmark

Hedgehogs are distributed throughout Denmark, although differences in local abundances may exist (Pagh 2007). Counts and estimations of roadkills in the 1970-90s showed a high number of hedgehogs, ranging between 50.000-80.000 per year in Denmark alone (Hansen 1982;

Jensen 1996; Laursen 1983; Madsen 1993). Further examinations of the population size of hedgehogs in Denmark have not been pursued, perhaps because hedgehogs are not included in the National Gamebag Census. Danish research on hedgehogs remains scarce, but the most significant contributions have been made by the late Professor Helge Walhovd and Anja Jensen, later Riber (Jensen 2004; Riber 2006). Furthermore, Sussie Pagh conducted a research project on hedgehogs and the invasive Portuguese slug (*Arion lusitanicus*) (Pagh 2004). Through several decades Danish hedgehogs have been represented in research on the parasitology of Danish wildlife (Borgpetersen & Fennestad 1962; Dietz et al. 1998; Enemark et al. 2002; Fennestad & Borg-Petersen 1972; Nauerby et al. 2000; Nielsen et al. 1978; Settnes et al. 1986). My master's thesis examined the ecology of rehabilitated and wild, juvenile hedgehogs in 2013 (Rasmussen 2013), and during my research I also collected samples for measures of glucocorticoid levels in the hedgehogs studied. This data set was supplemented with samples from the hedgehogs studied in Manuscript I, but has yet to be prepared for publication.

Evidence for zoonotic transmissions between hedgehogs and humans

One of the thesis aims was to investigate the potential for zoonotic transfers between hedgehogs and humans. As hedgehogs seem to be increasingly inhabiting residential areas, and people enjoy feeding their local hedgehogs in food bowls containing cat food and water, which is regularly shared with their pets (Doncaster et al. 2001; Hubert et al. 2011; Pettett et al. 2017; van de Poel et al. 2015). The close contact between hedgehogs and humans provides a potential risk for zoonotic and anthroponotic pathogen transmission, the transmission of bacterial and viral infections, fungi and parasites, between hedgehogs and humans and vice versa. The risk is likely higher for the many hedgehog rehabilitators handling infected hedgehogs on a daily basis.

In a recent surveillance program for disease in free-living hedgehogs in the UK, researchers found evidence of several zoonotic pathogens in deceased hedgehogs, such as *Listeria monocytogenes*, *Cryptosporidium parvum*, *Salmonella enteritidis*, and multiple herpesviruses (Hydeskov et al. 2019; Hydeskov et al. 2018; Lawson et al. 2018; Sangster et al. 2016). Among the detected herpesviruses in the hedgehogs was the *Human alphaherpesvirus 1*, which the infected hedgehogs likely contracted from humans, whilst being admitted to a wildlife rehabilitation center. The results found in the research on *S. enteritidis* were consistent with hedgehogs acting as a source of zoonotic infection in humans (Lawson et al. 2018). Furthermore, hedgehogs have previously been suspected a source of human outbreaks of

Salmonella infections in Denmark and Norway (Handeland et al. 2002; Nauerby et al. 2000), and Rautio et al. (2016) found a 57 % prevalence of *S. enteritidis* (phage type 20) in 37 dead, Finnish hedgehogs.

A study from Belgium concluded that hedgehogs contribute to the spread and transmission of tick-borne pathogens in urban areas, with a high prevalence of *Borrelia burgdorferi* genospecies (*Borrelia bavariensis*, *Borrelia spielmanii*, *Borellia afzelii*), *Anaplasma phagocytophilum* and *Rickettsia helvetica* in the two species of ticks, *Ixodes hexagonus* (n = 1132) and *Ixodes ricinus* (n = 73), found on 54 rehabilitated hedgehogs residing in gardens or parks in urban areas. Since humans share habitats with hedgehogs, they are likely to encounter ticks infected with these pathogens whilst gardening or recreating in the parks. In total, *Borrelia miyamotoi*, *A. phagocytophilum*, *R. helvetica*, and *B. burgdorferi* genospecies (*B. afzelii*, *B. bavariensis* and *B. spielmanii*) were found in the ticks (Jahfari et al. 2017). In a study of tick-borne pathogens and pathogenic bacteria and parasites with zoonotic potentials in fecal samples from rehabilitated and wild-living hedgehogs in the Netherlands, researchers found *A. phagocytophilum* and *Borrelia* genospecies *B. afzelii*, *B. spielmanii*, *B. garinii*, and *B. burgdorferi* in both *I. hexagonus* and *I. ricinus* ticks (n = 628) (Krawczyk et al. 2015). Through analyses of the faecal samples from 90 hedgehogs, the researchers detected *Salmonella enterica*, *Campylobacter jejuni*, as well as ESC-resistant *Escherichia coli*, along with the zoonotic protozoan parasites *Giardia duodenalis*, *Cryptosporidium parvum* and *Cryptosporidium hominis* (Krawczyk et al. 2015). Skuballa et al. (2012) used PCR amplification to test for *B. burgdorferi* in ticks sampled from hedgehogs from Germany (n = 211), Austria (n = 4), the Czech Republic (n = 22) and the UK (n = 32). *B. burgdorferi* prevalences ranged from 0 % (UK) to 37.5 % (Czech Republic) representing the *B. burgdorferi* genospecies, *B. afzelii*, *B. bavariensis* and *B. spielmanii*. Enemark et al. (2002) studied the genetic polymorphisms in 271 Danish *Cryptosporidium* isolates of human and animal origin, of which one was obtained from a hedgehog. The hedgehog isolate had a distinct sub-genotype compared to the other detected isolates.

Franklinos et al. (2015) reported the detection of a *Streptococcus pyogenes* infection from a carcass of a hedgehog in the UK. They hypothesised that this was caused by an anthroponotic infection because the strain identified (*emm* 28) is associated with human infections.

Furthermore, Hofmannova & Jurankova (2019) found DNA from the zoonotic parasite *Toxoplasma gondii* in the brain tissues of 5 *Erinaceus europaeus* (19.2 %) from the Czech Republic. Finally, the zoonotic potential of hedgehogs as a natural reservoir for *mecC*-MRSA is discussed in manuscript III of this thesis. With the increased focus during recent years on

zoonotic pathogens in wildlife, it is important to remember that carriage of e.g. resistant strains of *Staphylococcus aureus* in hedgehogs was already observed in the 1960's by Smith & Marples (1964). The modern-day technological development enables us to detect the pathogens more efficiently, even though they have probably been present in wildlife for decades at least.

In conclusion, there is substantial evidence for hedgehogs as reservoirs of a range of pathogenic zoonoses, and several case reports indicate anthroponotic transmission of infection to hedgehogs. However, the exact role and impact of the hedgehog as a source of human disease outbreaks remains to be quantified, and there is a tendency for wildlife being incorrectly incriminated as a reservoir for human infection. The potential for zoonotic transmission is undoubtedly present due to the shared habitats, which often result in direct and indirect contact between humans, domestic animals, and hedgehogs.

Genetics

Manuscript II of the thesis describes the genetic structure of Danish hedgehogs. This section therefore provides an introduction to the basic concepts of genetics, how the discipline of genetic research can be used for conservation purposes, and a summary of previous research on hedgehog genetics.

Introduction to population genetics

Small and isolated populations are more vulnerable to external environmental disturbances (environmental stochasticity) and incidental fluctuations in their local survival and fecundity (demographic stochasticity) (Keller & Waller 2002). Genetic drift, the change in the allelic frequency of a population generated by a random selection of genes, causes alleles to become randomly fixed or lost from the small and isolated populations, which leads to the loss of the genetic variation that is necessary for adaptive evolution. Inbreeding is the mating between closely related individuals. The homozygosity created by inbreeding often causes reduced fitness (inbreeding depression), as deleterious mutations will accumulate in the population (Keller & Waller 2002).

The Hardy-Weinberg principle, which population genetics is founded on, states that in a large, randomly mating population, which has no overlapping generations, the frequencies of genotypes at one diploid locus are the product of the allele frequencies at that locus and remain in a constant equilibrium between generations, on the condition that migration, mutation and natural selection do not affect that locus. A further assumption is that allele frequencies are the

same in males and females. Deviations from the Hardy-Weinberg equilibrium can be generated by any fluctuation from these assumptions, especially the lack of random mating, causing a loss of heterozygosity (Garnier-Géré & Chikhi 2013).

The Wahlund effect is defined as the surplus of homozygotes and the lack of heterozygotes observed in a large group of individuals due to the existence of population subdivision (Garnier-Géré & Chikhi 2013). A genetic bottleneck is the reduction of a population size due to environmental or anthropogenic events, like disease, floods, hunting, which reduces the variation in the gene pool of a population. The founder effect is defined as a form of genetic bottleneck caused by the isolation (geographically or reproductively) of a small group of individuals from the general population, which occurs through a founder event, such as the invasion of a new area by a fraction of the original population, the colonization of an isolated habitat, or individuals isolated from potential mates in e.g. a zoo setting (Provine 2004).

The effective population size, N_e , is defined as the number of individuals in an ideal population that provides the same rate of random genetic drift as the actual population. Simplified, it is the number of breeding individuals in a population. Factors such as unequal sex distribution, varying family size (litter size), and temporal fluctuations in population size, are the primary elements causing the effective sizes of natural populations to be smaller than the true population size. When none of the factors acting to maintain genetic variations are present, such as mutation, immigration, or selection favouring heterozygotes, the expected rate of heterozygosity loss is $1/(2N_e)$ per generation (Lande 1988). The Allee effect is the threshold density or number of individuals below which the population cannot recover, due to the decline in individual fitness at a low population size (Lande 1988).

Conservation genetics

The loss of suitable habitats and genetic diversity are important factors, which could lead to the extinction of wildlife populations or even whole species (Keller & Waller 2002; Lacy 1997). Preserving the genetic connectivity across fragmented landscapes is a vital tool to counteract the negative consequences of genetic drift and inbreeding. Conservation genetics as a discipline, including genetic rescues, is an effective method for assessing, conserving, and managing fragmented and genetically vulnerable populations (Ralls et al. 2018).

It is discussed whether inbreeding and loss of genetic variability should be considered a threat to a population's survival (Keller & Waller 2002). The major argument against it states that populations experiencing bottlenecks or other forms of strong inbreeding should be under

selection due to the exposure of deleterious recessive mutations, which will eventually reduce the inbreeding depression (Keller & Waller 2002). In other words, if inbreeding is so severe that certain hereditary and deadly diseases appear in the population, the inflicted individuals will not be able to breed anyway, and the diseases will not spread further in the population. Attributing the cause of death to genetic defects in wild populations is complex and rarely detectable. However, measuring how inbreeding makes individuals more vulnerable to environmentally induced mortality has been practiced for years. For example, a significant inbreeding depression in Soay sheep (*Ovis aries*), caused parasite-induced deaths, because more homozygous individuals suffered higher rates of parasitism and, in turn, lower overwinter survival compared to the heterozygous sheep (Coltman et al. 1999). Moreover, evidence for the negative effects of inbreeding on population dynamics can be clarified when new individuals from a genetically diverse population are introduced to inbred populations, experiencing low reproductive success due to the low genetic variability. As an example, twenty male adders (*Vipera berus*), were relocated to an inbred population with many deformed and stillborn offspring in southern Sweden. Subsequently, the fitness and population size were restored after the introduction of the genetically diverse males (Madsen et al. 1999). However, some populations of species seem to survive even with very low genetic heterozygosity. Research into the genetics of the Danish population of European otters (*Lutra lutra*) indicated a drastic decline around two thousand years ago, which left the genetic variability very low in the viable modern-day population (Pertoldi et al. 2001a). Furthermore, a study on five viable populations of Danish badgers (*Meles meles*) detected very low genetic heterozygosity within populations, which is likely caused by landscape fragmentation (Pertoldi et al. 2001b). However, more recent studies on badger populations in the UK indicate negative effects of inbreeding. Benton et al. (2018) showed that inbreeding intensifies a positive correlation between age and progressed infection of bovine tuberculosis in females. Annavi et al. (2014) found that badger cubs with heterozygous fathers had a higher first-year survival probability than cubs with less heterozygous fathers, but this tendency was only seen in years with higher summer rainfall.

The question remains whether there actually is a general pattern explaining how inbreeding depression influences population dynamics, or if it varies among taxa, habitats and populations with different demographic and genetic histories (Keller & Waller 2002).

One way of counteracting the negative effects of inbreeding in small, vulnerable populations is the attempt of genetic rescue, during which a gene flow is provided from another population of the same species (Frankham 2015). In a meta-analysis by Frankham (2015), the outcrossing

of inbred populations had beneficial effects in 92.9 % of 156 cases studied, with a higher fitness found in 148 % of species living under stressful conditions and 45 % of species living under stable conditions. There is, however, a risk of spreading parasites and disease by mixing separated populations, and the social structure of the population may be disrupted by newcomers. There is currently no clear guidelines for genetic rescues and the financial costs can be high, which may explain the rather low number of attempts at this practice so far (Frankham 2015).

Hedgehog genetics

Compared to other mammalian species, hedgehogs seem to lack a clear dispersal phase and they seldom cover distances larger than 4 km (Morris & Reeve 2008). The fragmentation of landscape caused primarily by roads is a barrier, reducing the movement of hedgehogs in the landscape, mainly due to roadkill (Doncaster et al. 2001; Dowding et al. 2010a; Huijser & Bergers 2000; Rautio et al. 2016; Rondinini & Doncaster 2002). However, habitat connectivity is vital for the movement and dispersal of animal species such as the hedgehog because it enhances the gene flow, which causes potential benefits for fitness, adaptability and survival of local populations (Braaker 2017; Reed 2004). Even though habitat fragmentation is most common in cities (Grimm et al. 2008), species such as the hedgehog can use the green spaces within the cities as an important network of habitats, thus forming a connective web within the urban matrix and potentially maintaining an amount of gene flow across the city (Hof & Bright 2009).

The promiscuity and heteropaternal superfecundation in hedgehogs (Moran et al. 2009) may have a positive impact on inbreeding, since a litter can contain half-siblings instead of full siblings (Moran et al. 2009). At the present time it remains unknown whether hedgehogs can actively differentiate between kin and non-kin, e.g. during the mating season. However, if there is no distinction between relatives and non-relatives when choosing mates, small isolated populations of hedgehogs will easily become even more vulnerable due to the increased degree of inbreeding. Therefore, it is highly relevant to study the hedgehogs at a molecular level and estimate the conservation status of the different hedgehog populations.

Previous genetic research on hedgehogs has been based on the use of microsatellites (Barthel 2019; Becher & Griffiths 1997; Becher & Griffiths 1998; Berggren et al. 2005; Bolfikova & Hulva 2012; Bolfikova et al. 2013; Bolfikova et al. 2017; Braaker 2017; Fraser et al. 2012; Henderson et al. 2000; Moran et al. 2009; O'Reilly 2016; Santucci et al. 1998; Seddon

et al. 2001). Research into the glacial refugia and interglacial expansion of the European hedgehog showed three monophyletic clades (Seddon et al. 2001). The first from Italy northwards through Austria, Switzerland, Germany, the Netherlands, Scandinavia and Estonia. The second was only found in western Europe, from Spain northwards through France, the Netherlands and into the UK and Ireland. The third clade was restricted to Sicily (Seddon et al. 2001).

Bolfikova & Hulva (2012) described the population structure and patterns of gene flow in *E. europaeus* and *E. roumanicus* inhabiting the central European contact zone between the two species. They discovered that the homogenous population of *E. europaeus*, had a significantly lower observed heterozygosity (H_O) than expected heterozygosity (H_E) in five of the nine microsatellite loci studied, with a mean H_O of 0.695 and mean H_E of 0.6872 ($n = 131$). The population had been divided into a subpopulation in the western part, and two subpopulations with a mosaic location pattern in the eastern part of the country, by two large rivers in the Czech Republic (Vltava and Elbe). Research conducted on 42 individuals in an isolated population of hedgehogs in the Regent's Park, London UK, concluded that the population had a very low genetic diversity, with a mean H_E of 0.197, and a mean H_O of 0.198 ($n_{\text{loci}} = 6$) (O'Reilly 2016). A clear restriction of gene flow was detected between eight small populations of hedgehogs in a 15 km² fragmented landscape in Oxfordshire, UK by Becher & Griffiths (1998). They observed a statistically significant genetic differentiation among the studied populations ($n = 160$, $n_{\text{loci}} = 6$) and a mean H_O of 0.7. Human-mediated barriers such as roads and train tracks had fragmented the landscape, thereby restricting the dispersal of hedgehogs and hence the gene flow between the isolated populations (Becher & Griffiths 1998). Braaker (2017) studied the spatial genetic structure of 147 hedgehogs inhabiting Zurich, Switzerland, with an area of 88 km² ($n_{\text{loci}} = 10$). The population of hedgehogs in Zurich consisted of three genetic clusters, separated by two rivers and the major roads. Mean H_E ranged between 0.569-0.627 for the three clusters, and the mean H_O ranged between 0.523-0.631, and the inbreeding coefficients (F) were low (Braaker 2017). Recently, Barthel (2019) genotyped 143 individuals throughout Berlin ($n_{\text{loci}} = 10$) and found three genetic clusters with $H_O = 0.557-0.623$ and $H_E = 0.524-0.685$. Two of the clusters were based in two different parks in Berlin, but the main genetic cluster was very widespread and also contained individuals living in the areas of the other clusters. Barthel (2019) hypothesised that the main reason for the apparent high gene flow is the large proportion of green areas in Berlin, connecting suitable habitats across the city.

What remains to be investigated is the extent to which a low individual genetic heterozygosity negatively influences hedgehog fitness and survival. Hopefully, the final results from The Danish Hedgehog Project will eventually elucidate this, by comparing genetic heterozygosity with measures of e.g. parasitic load, dental health and the prevalence of cancer.

MRSA

Manuscript III describes the detection of methicillin-resistant *Staphylococcus aureus* (MRSA) in Danish hedgehogs. This section will provide a general introduction to bacterial resistance, MRSA and the risks relating to the spread of MRSA, and finally present an overview of previous studies on MRSA in hedgehogs.

Bacterial resistance

Bacterial resistance is developing rapidly and is occurring worldwide, threatening to abolish the effects of antibiotics, which have altered medicinal practices and saved countless lives, since the first discovery of penicillin by Sir Alexander Fleming in 1928 (Ventola 2015). Today, bacterial infections have once again reached a potential to become life-threatening, even in developed countries with access to medical care. The cause of the modern-day antibiotic resistance crisis lies in the excessive consumption and improper use of the drugs, and a neglect by the pharmaceutical industry to develop new antibiotic drugs (Lushniak 2014). The Centers for Disease Control and Prevention (CDC) and The World Health Organization (WHO) have stated that antibiotic resistance is one of the biggest public health challenges of our time. CDC has categorized a number of bacterial species as being of urgent, serious, and concerning threat to public health (Centers for Disease Control and Prevention 2013). Methicillin-resistant *Staphylococcus aureus* is one of them.

Methicillin-resistant *Staphylococcus aureus*

Staphylococcus aureus is a widespread, opportunistic pathogen with the potential to cause lethal infections in humans, especially due to the development of antibiotic resistance. *Staphylococcus aureus* was first identified by Sir Alexander Ogston in 1880, where it was isolated from infected surgical wounds (Ogston 1881). Roughly 30 % of the human population carries *S. aureus* as a commensal organism in e.g. the nasopharynx, throat or intestinal tract, since *S. aureus* primarily colonises skin and mucosae (Wertheim et al. 2005). *Staphylococcus aureus* can become pathogenic and cause a wide range of potentially life-threatening

infections, such as infective endocarditis, necrotizing pneumonia and osteomyelitis (Tong et al. 2015). The DNA-sequence-based typing technique, multilocus sequence typing (MLST), has been used to categorize *S. aureus* into several biotypes with more than two thousand sequence types (STs) identifying a large number of host-specific strains of *S. aureus* in the MLST database (Shepherd et al. 2013). The MLST subtypes (ST types) are furthermore divided into related families called clonal complexes (CC) (Shepherd et al. 2013).

Since the first description of methicillin-resistant *Staphylococcus aureus* (MRSA) in 1961 (Jevons 1961), the increasing incidence and spread of MRSA continues to cause extensive challenges for infection prevention, control and antibiotic treatment around the globe. More than 150,000 patients are annually infected with MRSA in the European Union (EU) alone (Kock et al. 2010). Due to the presence of penicillin binding proteins (PBPs) with low affinity to β -lactams, MRSA is resistant to most β -lactam antibiotics. The PBPs are encoded by either *mecA* or *mecC* genes carried in mobile genetic elements, called Staphylococcal Cassette Chromosome *mec* (SCC*mec*) (Paterson et al. 2014a). MRSA is the dominant cause of hospital-associated infections in Europe (Kock et al. 2010), and since the 1990s, a rise in community-associated MRSA infections among people with no apparent risk factors for contracting MRSA, has been observed (Aires-de-Sousa 2017). Furthermore, during the last decade, livestock-associated MRSA (LA-MRSA) has been confirmed as a third major cause of MRSA infections in humans (Graveland et al. 2011; Kock et al. 2010; Petersen et al. 2013a).

The production of pigs and pig meat is a major source of income for Denmark. Almost 90% of the production is exported, contributing markedly to the Danish economy and balance of trade. In July 2018 there were approximately 12,9 million pigs in Denmark (Statistics Denmark 2018). In 2006, the LA-MRSA carrying the *mecA* gene (CC-type 398) was first identified in Danish slaughter pigs (Guardabassi et al. 2007). A Danish national survey from 2008 found that 3.5 % of the Danish pig production herds were LA-MRSA CC398 positive (EFSA 2009). In 2016, the prevalence had increased to 88 % (DANMAP 2016). The prevalence of LA-MRSA CC398 is, so far, lower in other categories of production animals in Denmark, ranging from 2-16 % in poultry farms, organic pig farms, veal calf farms and mink farms (DANMAP 2015). However, the spread of LA-MRSA CC398 infections to people not working with livestock has increased in the recent years, and it has furthermore been observed that LA-MRSA CC398 is transmitted through handling and consumption of contaminated food and food products, such as meat from pigs, turkeys and chickens (Larsen et al. 2015; Larsen et al. 2016). The increasing rate of LA-MRSA CC398 infections in people with no livestock exposure is particularly

worrying, seeing as a high proportion of these patients are elderly and immunocompromised individuals, and consequently at a high risk of developing severe or even lethal staphylococcal illnesses (Larsen et al. 2015).

The *mecA*-MRSA is exceedingly causing most human MRSA infections. However, in 2011 researchers first described a hitherto unknown resistance gene, the *mecC* gene, in *S. aureus* from humans and dairy cattle from Denmark, England, Ireland and Scotland (Garcia-Alvarez et al. 2011; Shore et al. 2011). Consequently, *mecC*-MRSA was isolated from a broad array of domesticated animals from Europe, such as swine, small ruminants and horses in Denmark (Angen et al. 2017; Eriksson et al. 2013; Islam et al. 2017), as well as British cattle (Paterson et al. 2014b). It was originally believed that *mecC*-MRSA had a livestock reservoir, but after the isolation of *mecC*-MRSA in a vast range of species of European wildlife (Aires-de-Sousa 2017; Bengtsson et al. 2017; Carson et al. 2012; Gomez et al. 2014; Gomez et al. 2015; Himsworth et al. 2014; Loncaric et al. 2014; Monecke et al. 2016; Monecke et al. 2013; Mrochen et al. 2017; Porrero et al. 2013; Porrero et al. 2014), as well as urban waste water and river water (Concepcion Porrero et al. 2014), the theory was rejected. It was later suggested by Monecke et al. (2013) that *mecC*-MRSA could have evolved in wildlife. Zoonotic transmission of *mecC*-MRSA from livestock to humans is steadily observed (Goerge et al. 2017; Harrison et al. 2013; Paterson et al. 2013; Petersen et al. 2013a). *mecC*-MRSA is found in 1-2 % (30-50 cases annually) of all human cases of MRSA infections in Denmark, primarily causing skin and soft tissue infections (Petersen et al. 2013b).

MRSA in European hedgehogs

Hedgehogs prefer to inhabit areas associated with human activity, such as gardens in residential areas and rural villages (Hubert et al. 2011; van de Poel et al. 2015). As already described, cat food and water is often made available by homeowners for hedgehogs residing in suburban gardens as a way of attracting and helping the hedgehogs (Morris 2014). Living side by side, there is a tendency for hedgehogs to become habituated to human presence, which makes the hedgehog one of the few wild mammals people are prone to come into physical contact with. This, alongside the potential sharing of food and water bowls with the garden owners' pets, poses a potential risk for zoonotic transmission between hedgehogs and humans.

Penicillin-resistant *S. aureus* in hedgehogs was first described in 1964 (Smith & Marples 1964). Smith & Marples (1964) isolated *S. aureus* in 40 % of the nasal swabs, 63 % of the skin surface swabs and 71 % of the paw swabs, which were sampled from 35 hedgehogs in New

Zealand. Between 79-92 % of these *S. aureus* isolates were resistant to penicillin. Additionally, Smith (1965) found a prevalence of *S. aureus* of 85 % in 59 hedgehogs from New Zealand, and detected penicillin resistance in 86 % of these isolates. However, it is unknown whether the isolates were actual *mecC*-MRSA, because the first finding of a confirmed *mecC*-MRSA case was not until 1975 (Garcia-Alvarez et al. 2011).

Two studies have confirmed that European hedgehogs from Sweden carry *mecC*-MRSA (Bengtsson et al. 2017; Monecke et al. 2013). Monecke et al. (2013) described how the detected *mecC*-MRSA caused severe or even lethal infections in the two hedgehogs studied, and Bengtsson et al. (2017) found a high prevalence of 64 % *mecC*-MRSA in 55 hedgehogs from five counties in Sweden, out of which 49 individuals died in care at wildlife rehabilitation centres.

Compared to other wildlife species, hedgehogs seem to have a very high prevalence of *mecC*-MRSA carriage. Gomez et al. (2014) isolated *mecC*-MRSA in 2 % of 101 faecal samples from six small mammal species in Spain, and found a 17 % prevalence in 65 nasal swabs from farmed red deer in Southern Spain (Gomez et al. 2015).

Smith & Marples (1965) and Bengtsson et al. (2017) have all suggested a possible connection between the high MRSA prevalence found in hedgehogs and the presence of dermatophytes in hedgehogs producing penicillin-like substances (*Trichophyton mentagrophytes* var. *erinacei*), potentially providing a fitness advantage for *S. aureus* to become methicillin-resistant. This connection is currently being investigated by the present author and collaborators in Denmark and the UK.

Summary of manuscripts

The overall aim of this thesis is to investigate specific ways humans influence hedgehogs when sharing habitats and living closely together (Manuscripts I and II), as well as describe how hedgehogs may affect humans, in this case through potential zoonotic transmission of *mecC*-MRSA (Manuscript III). The focal points for the studies of the anthropogenic effects on hedgehogs are, 1) a description of the ecology of juvenile hedgehogs living in suburban habitats consisting primarily of residential gardens, and 2) the genetic structure of hedgehogs in Denmark, which is influenced by anthropogenic factors such as habitat fragmentation and traffic, as well as the translocation of hedgehogs by rehabilitators and ordinary citizens.

Manuscript I: The ecology of suburban juvenile European hedgehogs (*Erinaceus europaeus*) in a changing Danish climate.

The aim of this study was to describe the ecology of juvenile hedgehogs residing in suburban habitats. We strived to fill the existing knowledge gap on suburban-living juvenile hedgehogs by reporting home range size estimates, survival, weight development and hibernation behaviour during their first year of life, from September 2014-July 2015. Our objective was to improve the understanding of the challenges faced by suburban-living hedgehogs, thereby providing insight, which could benefit future work to design and target conservation initiatives for hedgehogs living in the suburbs.

We used radio-tracking data to estimate the home range sizes of male and female juvenile hedgehogs in autumn and spring/summer, their survival during their first year of life, their weight changes before, during and after hibernation, and the hibernation behaviour of the juvenile hedgehogs. We furthermore observed how local climatic conditions influenced the behaviour of the hedgehogs. We also reported how human recklessness caused mortalities in the studied population, through injuries with garden tools and loose dogs, and how a *Salmonella* infection spread in a local population, through transmission from food and water bowls shared by several hedgehogs in a garden.

Conducting this long-term study (September 2014-July 2015) on 35 individuals, we found that the radio tracked males and females had small home ranges compared to previous studies, indicating adequate food and nest availability in the suburban habitats. The juvenile survival probabilities during the study period were high compared to previous research on juvenile hedgehogs. We found that all healthy individuals gained weight during the autumn and survived hibernation with little weight loss, thus demonstrating that they were capable of gaining sufficient weight in the wild to survive their first hibernation. Additionally, we described how the exceptionally mild autumn of 2014 caused the hedgehogs to delay hibernation for up to a month compared to previous studies in Denmark and we made the first records of second litters being born in Denmark.

Manuscript II: Genetic structure of the European hedgehog (*Erinaceus europaeus*) in Denmark.

We aimed to describe the genetic structure of a nationwide sample of hedgehogs from Denmark by adapting the second-generation genotyping technique, genotyping by sequencing (GBS), for the genetical analysis of European hedgehogs. We intended to provide a set of SNPs which could be applied for research into the genetic structure and variability of the European hedgehog on a broader scale, in order to assess the genetic diversity distribution of the Danish hedgehog populations and to describe any possible the historical changes in their effective population size (N_e) by use of genetic signatures.

We analysed samples from 178 individuals from six geographically and genetically distinct populations (Jutland north of the Limfjord, Jutland south of the Limfjord, Funen, Zealand, Falster and Lolland, Bornholm) and estimated inbreeding coefficients, the observed, expected and unbiased expected heterozygosity, and the percentage of polymorphic loci. We tested for deviations from Hardy-Weinberg equilibrium (HWE) and for patterns of isolation by distance (IBD). Additionally, we determined the genetic structure of the individuals based on a Bayesian clustering method and tested for recent changes in population size.

We found that the Danish hedgehog population could be divided into three genetic clusters: 1) Jutland north of the Limfjord (JNL) and Jutland south of the Limfjord (JSL), 2) Funen (F), Zealand (Z), Lolland and Falster (LFA) and 3) Bornholm (BH).

Compared to previous studies on hedgehog genetics, we found a low genetical heterozygosity in the Danish hedgehogs. The heterozygosity deficiency was reflected in the positive inbreeding coefficients calculated for five of six populations, and a significant deviation from HWE in three of six populations tested, which indicated inbreeding. The Mantel test for association between genetical and geographical distance within populations showed that there were no significant differences in the genetic structure within the otherwise genetically distinct six populations in the study.

We also tested for possible anthropogenic effects on the genetic variability of the hedgehogs, particularly the effects of densities of human populations, roads and farmland, and found that inbreeding decreased with increasing farmland density. This was surprising, as we had expected to find more severe inbreeding in areas with many roads and a high human population density as indicators of habitat fragmentation.

The significant and positive inbreeding coefficients we detected in the majority of the sampled Danish hedgehogs can be caused by inbreeding, genetic substructure and low effective population size, which are all consequences of habitat fragmentation. We could not detect signs of a recent population bottleneck and/or population increase or decline. Nonetheless, we were unable to reject the possibility of a longer-term decline in the Danish hedgehog population because the available test could only determine very recent changes in population size.

Manuscript III: European hedgehogs (*Erinaceus europaeus*) as a natural reservoir of methicillin-resistant *Staphylococcus aureus* carrying *mecC* in Denmark.

The objective of this study was to determine the prevalence, distribution and type of methicillin-resistant *Staphylococcus aureus* (MRSA) in Danish hedgehogs. We aimed to investigate whether the high MRSA prevalence found in Swedish hedgehogs could also be detected in Denmark, and to determine if hedgehogs contribute to the zoonotic spread of MRSA to Danish citizens, as hedgehogs travel everywhere and often come into direct or indirect contact with humans through the sharing of habitats.

As previous studies on MRSA in wildlife have been limited by factors such as small sample sizes, small geographical ranges, or the use of samples from weak animals that have been in close contact with other wildlife species and humans during care at wildlife rehabilitation centres, we wanted to improve these limitations by using our samples from a large-scale nationwide sampling of wild hedgehogs in Denmark. Our large data set represented the hedgehog population of a whole country, as well as a broader spectrum of individuals, since the collected hedgehogs in our study had not only died in care at hedgehog rehabilitation centres, but also in the wild from natural causes or due to roadkill accidents.

We obtained nasal swabs from 188 dead hedgehogs collected by volunteers throughout Denmark, to determine the prevalence of MRSA. Additionally, we tested 16 hedgehog rehabilitators to estimate the potential zoonotic transmission of MRSA from hedgehogs to humans.

We found that a total of 114 (61 %) hedgehogs carried *mecC*-MRSA. The detected *mecC*-MRSA came from two genetic lineages CC130 and CC1943 and 12 different *spa*-types. The prevalence of *mecC*-MRSA in the hedgehogs was similar regardless of cause of death, sex and habitat type, or region.

Our research showed a high prevalence of *mecC*-MRSA in Danish hedgehogs, suggesting that hedgehogs could serve as a natural reservoir for this specific type of MRSA. We found no sign of zoonotic transmission of *mecC*-MRSA to hedgehog rehabilitators.

Discussion and future perspectives

With the accumulating scientific evidence for the decline of the European hedgehog in western Europe, it is essential that we gather knowledge to direct and optimise conservation efforts leading to the preservation of the species in the wild.

The research presented in this thesis clearly indicates that anthropogenic influences do affect hedgehogs in Denmark, and it furthermore shows that there is a risk for zoonotic transmission between hedgehogs and humans when living closely together. The best way to avoid or lessen the negative influences humans and hedgehogs exert on each other, must be to educate the citizens about the challenges hedgehogs face when co-existing with humans. As we are probably not in a position to reform the modern-day agricultural practices, the conservation effort must be directed at improving the quality of the habitats associated with human presence, such as the rural villages, as well as the urban and suburban environments. Information on e.g. how to make gardens hedgehog friendly, how to enhance garden connectivity, and thereby facilitate the movement of hedgehogs in the suburban environment and increase the gene-flow and persistence of sustainable populations, should be made available to garden owners everywhere. Encouraging architects and contractors to consider urban-living wildlife when planning and carrying out new construction work, may also increase the quality of urban habitats for a range of wildlife species. Danes are generally very fond of hedgehogs, and the enthusiasm and participation in the Danish Hedgehog Project implies that there is a sincere wish to make a difference for the hedgehogs. However, the general lack of education for hedgehog rehabilitators and garden owners on how to properly and hygienically handle and care for hedgehogs to avoid zoonotic transfers and inexperienced treatment of hedgehogs, as well as the constant flow of well-meant fake news about hedgehogs in the media, is really not beneficial for the hedgehog conservation. The Danish authorities have recently established legal frameworks and monitoring programs for the practice of wildlife rehabilitation, which is a long-awaited step in the right direction.

Research and monitoring of hedgehogs in Denmark has been neglected, and we lack conservation monitoring programs and educational programs like the British initiatives Garden Wildlife Health and Hedgehog Street, with permanent employees responsible for organising

the citizen science initiatives and communicating to the public, in addition to raising funds and providing funding for the research produced. The potential for citizen science programs in Denmark is huge, especially when the goal is to preserve a well-liked species like the hedgehog in the wild.

The assembled results from the Danish Hedgehog Project will contribute markedly to the understanding and evaluation of the conservation status of the Danish hedgehog population, and hedgehogs in general, especially due to the unprecedentedly high nationwide sample size and the diversity of the research being performed. During the next years, if funding is made available, the results from the collected samples will be obtained, compiled, and analysed, thereby providing solid background knowledge on the health and conservation status of the Danish hedgehogs. Altogether, the already finished and the future research of the project includes genetics, MRSA detection, parasitology, dental health and age determination studies, gut microbiome characterisation, toxicology, morphometrics, diet choice analyses, population modelling, definition and description of roadkill hot spots, *Salmonella* detection and histopathology (including cancer research). Additionally, the remaining kidney, spleen and tissue samples will eventually be devoted to the most relevant fields of research. The knowledge gained will enable us to build advanced population models predicting the expected development in population size of Danish hedgehogs. Furthermore, it is my hope that the research could be applied in a broader perspective benefitting even more species, with hedgehogs functioning as a model species for smaller mammals. An example of this could be our detailed quantification of the health effects of inbreeding, for which scientific evidence is deficient for a wide range of species.

Many unanswered questions remain regarding the cause of the decline in hedgehogs, but hopefully the enhanced focus on hedgehog conservation, which initiatives like the Danish Hedgehog Project and Hedgehog Street have generated via the media and the public, will help procure funding for a range of future research, educational programs and conservation projects dedicated to the preservation of hedgehogs in the wild.

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Manuscript I

The ecology of suburban juvenile European hedgehogs (*Erinaceus europaeus*) in a changing Danish climate



The ecology of suburban juvenile European hedgehogs (*Erinaceus europaeus*) in a changing Danish climate

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Abstract

1. European hedgehog (*Erinaceus europaeus*) populations are widespread across diverse habitats but are declining in western Europe. Drastic declines have been described in the UK, with the most severe declines occurring in rural areas. Hedgehogs are widely distributed in Denmark, but their status remains unknown. Arable land is thought to have become particularly unfavourable due to agricultural intensification and habitat fragmentation.
2. Fieldwork on hedgehogs has tended to focus on rural areas, leaving their ecology in suburban habitats largely unexplored, with clear implications for conservation initiatives. Here we study the ecology of 35 juvenile hedgehogs using radio tracking during their first year of life in the suburbs of western Copenhagen.
3. We use radio-tracking data to estimate (a) home range sizes in males and females in autumn and spring/summer, (b) survival during their first year of life from September 2014-July 2015, (c) the weight changes before, during and after hibernation, and (d) the hibernation behaviour of the juvenile hedgehogs.
4. We show that males and females have small home ranges compared to previous studies (95 % MCP home range: autumn 1.36 ± 0.24 ha (mean \pm SD, $n = 15$) and spring/summer: 5.1 ± 4.85 ha (mean \pm SD, $n = 7$)), indicating adequate food and nest availability. The juvenile survival probabilities during the study period from September 2014-July 2015 were 0.56 for females and 0.79 for males. All healthy individuals gained weight during the autumn and survived hibernation with little weight loss thus demonstrating that the juveniles in the study were capable of gaining sufficient weight in the wild to survive their first hibernation.
5. Climate is changing, but there is a lack of knowledge on how this affects mammal ecology. The exceptionally mild autumn of 2014 caused the hedgehogs to delay hibernation for up to a month compared to previous studies in Denmark.

Introduction

There is substantial evidence that European hedgehog (*Erinaceus europaeus*) populations are declining based on monitoring data from the UK, Belgium, the Netherlands, Sweden, and Germany (Hof & Bright 2016; Holsbeek et al. 1999; Huijser & Bergers 2000; Krange 2015; Müller 2018; SoBH 2011; SoBH 2015; SoBH 2018; van de Poel et al. 2015; Williams et al. 2018). These declines are believed to be driven by habitat loss and fragmentation, intensified agricultural practices, road traffic accidents, molluscicide and rodenticide poisoning and in some areas badger predation (Brakes & Smith 2005; Dowding et al. 2010a; Dowding et al. 2010b; Haigh et al. 2012; Hof & Bright 2010; Huijser & Bergers 2000; SoBH 2011; Young et al. 2006).

The study of hedgehog ecology in urban areas is underrepresented in the literature, even though hedgehogs seem to prefer residential areas (Doncaster et al. 2001; Hubert et al. 2011; Pettett et al. 2017; van de Poel et al. 2015). This preference could be due to higher food densities affiliated with human occupation, including natural prey and anthropogenic sources, more suitable nest sites and a decreased risk of predation by badgers (*Meles meles*) (Micol et al. 1994; Morris 1985; Pettett et al. 2017; Young et al. 2006). In the UK, it is furthermore suggested that the hedgehog decline is currently more severe in the rural than urban areas (SoBH 2018; Williams et al. 2018). Since urban habitats may be more suitable for hedgehogs at the present time, it is relevant to describe the challenges hedgehogs face when living in this habitat type, to plan the optimal conservation initiatives directed at preserving hedgehogs in urban areas.

Previous studies on urban hedgehogs indicate that sheltered climatic conditions and anthropogenic food resources may be important predictors of increased presence of hedgehogs in urban areas compared to rural areas (Hubert et al. 2011). Dowding et al. (2010a) found that hedgehogs residing in urban areas primarily became active after midnight and avoided foraging near roads, likely to reduce the dangers and disturbances caused by human activities such as vehicle and foot traffic and the risk of predation by dogs (Morris & Reeve 2008; Reeve & Huijser 1999; Stocker 2005). Green spaces in urban areas, such as parks, road verges and gardens are often maintained thoroughly and may support several populations of wildlife e.g. amphibians and smaller mammals (Dickman 1987). However, the fragmentation of the suitable habitats caused by roads, water-bodies and impenetrable fences is a challenge for the survival and genetic diversity of the populations (Braaker et al. 2017; Hof & Bright 2009). Hof & Bright (2009) suggested that initiatives taken by garden owners to increase the attractiveness of their gardens for wildlife, by adding features such as nest boxes and feeders, may attract hedgehogs to gardens. However, garden habitat quality in residential areas is weakened by the use of garden pesticides (insecticides, molluscicides and rodenticides) which

reduces the availability of natural food items for the hedgehogs and may cause secondary poisoning (Ditchkoff et al. 2006; Dowding et al. 2010b).

Home ranges of wild juvenile hedgehogs

A home range is the spatial area in which an animal concentrates its activities in a defined time period, and is a useful tool for understanding the spatial ecology of a species and for optimising conservation initiatives (Spencer 2012). Juvenile hedgehogs (<12 months of age) tend to have smaller home ranges than adults (Kristiansson 1984a). When comparing measures of home ranges, it is important to consider the methodology used, such as the number of observations leading to the calculations, the study duration, habitat type, monitoring method (capture-mark-recapture (CMR) or radio/GPS tracking), number of fixes per night, calculation methods such as minimum convex polygons (MCP) and kernel density estimates (KDE), and the life stage of the monitored individuals (Morris 1988).

Using CMR, Kristiansson (1984a) calculated home ranges of 9.2 ha (sub-adult males) and 3.4 ha (sub-adult females) in a Swedish village. Reeve (1982) found home ranges of 10-15 ha during 83 nights of radio tracking (n = 3, aged 6-12 months) on a golf course in west London, UK. Sæther (1997) measured home ranges of 2.6-3.0 ha (MCP) by radio tracking (n = 15, aged 4-9 weeks) for 4 weeks in a residential area near Trondheim, Norway. Furthermore, Kristiansson & Erlinge (1977) reported a home range of 3.7 ha (n = 1, aged 3 months, October) and Rasmussen (2013) found home ranges of 3.54-4.85 ha (MCP) (n = 2, aged 3-4 months, October-November). In comparison, 10 adult hedgehogs were radio tracked in a rural area near Århus, Denmark in the summer of 2005, generating mean MCP home ranges of 96 ± 24 ha (mean \pm SD, males, n = 4) and 26 ± 15 ha (mean \pm SD, females, n = 4) (Riber 2006). Only the two studies from Denmark (Rasmussen 2013; Riber 2006) used GPS as a tool for registering the positions of the radio tracked hedgehogs. In conclusion, previously recorded home ranges of wild juvenile (up to 12 months of age) hedgehogs range between 2.6-15 ha.

Survival rates of wild juvenile hedgehogs in Scandinavia

The viability of a population is heavily influenced by the survival probability of the individuals in the population. A high survival probability of juvenile hedgehogs during their first year of life is important for the growth of the population because surviving juveniles will eventually join the breeding population. Reported survival probabilities of Scandinavian juvenile hedgehogs have varied widely depending on age, background (rehabilitated or wild), the time of year, habitat type, tracking method, location and climatic conditions in the study period (Jensen 2004; Kristiansson 1984b; Kristiansson 1990; Rasmussen 2013; Sæther 1997; Walhovd 1990). Sæther (1997) found an autumn survival probability of 0.31 for 25 radio-tagged juvenile hedgehogs (during their 4th to 9th week of life) in a residential area in Norway. Kristiansson (1990) estimated an average annual juvenile

survival of 0.66 (n = 123), using CMR in a Swedish village. A pre-hibernation survival probability of 0.50 was estimated for 10 wild, radio-tagged juveniles in a recreational area near Copenhagen, Denmark (Rasmussen 2013), while Jensen (2004) found a 100 % winter survival for seven Danish radio-tagged juvenile hedgehogs. In summary survival probabilities for Scandinavian hedgehogs range between 0.31-1.00 depending on the age and period of time in which they were studied.

Hibernation of juvenile hedgehogs in Scandinavia

Surviving their first hibernation is a challenge for juvenile hedgehogs. Survival is dependent on factors such as nest quality (Morris 1973), health (particularly the amount of fat deposits (Kristiansson 1990)), and weather during the hibernation period. The optimal pre-hibernation weight for juveniles to survive hibernation has been debated for years. Morris (1984) suggested a minimum weight of 450 grams in order to survive hibernation and studies on Danish juveniles also indicated that ≥ 450 grams would be sufficient (Jensen 2004; Rasmussen 2013).

Considering past research on hibernation weight and survival of juvenile hedgehogs in Scandinavia, Jensen (2004) found that following an average autumn temperature in 2001, six Danish rural-living juvenile hedgehogs all survived (513-897 grams pre-hibernation), using two-four nests. Walhovd (1990) recorded an average spring recapture rate (CMR) of 69 % among juvenile hedgehogs (400-800 grams pre-hibernation) in Denmark, during six years of study. Likewise, Kristiansson (1984b) estimated the winter survival in southern Sweden to be an average of 67 % for all age categories, based on CMR.

In summary, past studies on the hibernation of juvenile hedgehogs in Scandinavia found that the onset of hibernation is normally between late October and November, and activity is resumed from mid-April to mid-May, that juvenile hedgehogs change nests 0-4 times during winter and the winter survival ranges between 60-100 % (Jensen 2004; Kristiansson 1984b; Kristiansson 1990; Rasmussen 2013; Walhovd 1976; Walhovd 1978; Walhovd 1990).

The potential effects of climate change on hedgehogs

Understanding how climate change may affect hedgehog demography is essential for the conservation of the species. Anthropogenic climate change has already impacted wild species (Parmesan et al. 2013), shifting their geographic ranges and seasonal activities (Intergovernmental Panel on Climate Change 2014). Because weather conditions are believed to be one of the possible triggers for the onsets of the breeding season and the hibernation for hedgehogs (Morris 2018), it is likely that changes in weather patterns driven by climate change will influence their survival and reproductive output.

In hedgehogs, warmer winters could pose a particular risk, because the periodic rises in temperature (to $\geq 10^{\circ}\text{C}$) may induce arousals from torpor (Kristoffersson & Soivio 1964; Newman 2015), initiating the thermoregulatory responses that lead to increased metabolic rates and a rapid return of body temperature to the normal levels (Carey et al. 2003), which may increase the drain of fat reserves of the hedgehogs. Furthermore, drier seasons in general could limit the amount of food items such as earthworms, since their abundance and distributions are sensitive to microclimatic conditions such as soil moisture (Edwards & Bohlen 1996; Macdonald et al. 2010). The warmer and wetter conditions may also cause increasing viability, population sizes and biting rates of a range of disease vectors infecting hedgehogs such as ticks, potentially carrying Lyme disease (Gern et al. 1997; Harvell et al. 2002; Macdonald et al. 2014).

So far, research on the effects of climate change on hedgehog ecology remain sparse. A study on the effect of climate change on post-hibernation emergence of hedgehogs in the UK showed, that the emergence timings of hedgehogs do appear to be linked to variation in local climatic conditions (PTES & BHPS 2015). However, a general effect of climate change could not be found.

The status of the Danish hedgehog population and the aim of the research

Due to the lack of monitoring of hedgehogs in Denmark, their conservation status remains unknown. It is however likely, that the decline found in other European countries is similar in Denmark, which has comparable habitat fragmentation, landscape structure, farm management practices and climate to the relevant countries, from which the decline in hedgehog populations has been detected. Hopefully, the current ongoing research on the Danish hedgehog population will eventually enable an estimation of their conservation status.

The aim of this study is to describe the ecology of juvenile hedgehogs residing in suburban habitats during their first year of life. Specifically, we fill a knowledge gap that exists on suburban-living juvenile hedgehogs by reporting home range size estimates, survival, weight development and hibernation behaviour during their first year of life from September 2014-July 2015.

Materials and Methods

Home range size

To estimate home range size we radio-tagged and tracked 35 independent juvenile hedgehogs in the western suburbs of Copenhagen, Denmark from 20th September 2014 to 22nd July 2015. These areas are dominated by housing and private gardens and we obtained access permission via a local media advertising campaign. To increase trapping success, we placed a Bolyguard MMS 550M 8MP

wildlife camera in front of feeding stations situated in gardens, where juvenile hedgehogs were known to appear. The camera sent a picture to the smartphone of the researcher waiting outside the garden whenever movement was detected at the feeding station, enabling efficient capture while minimising disturbance. We attached the radio tags, (PIP or TW3 tags from Biotrack Ltd, weighing 3g and 11g respectively) to the hedgehog's spines using quick-drying two-part epoxy glue and, to aid recovery, we added reflective tape to the tags. Our radio transmitters weighed less than 5 % of the hedgehogs' body weight, in accordance with the guidelines of the American Society of Mammalogists (Sikes 2016). We released the animals after a clinical examination and weighing on a digital kitchen scale in a bowl which we sanitised with alcohol between uses. We tagged most animals (33) between 21st September and the 26th November 2014 and a further two post-hibernation in April 2015. A small number (n = 3) of the initial 33 tagged were rehabilitated, orphaned siblings. All tagged animals were independent from their mothers (>6 weeks of age) when entering the study and weighed between 213 and 659 grams when tagged.

During the autumn, spring and summer of 2014-15 we radio tracked these hedgehogs using a Sika receiver and Yagi antenna during their activity periods between sunset and sunrise. This work represented 70 nights of fieldwork in the autumn of 2014 and 84 nights of fieldwork during the spring and summer of 2015. Each night we made position estimates for the animals with one hour intervals and we recorded the positions using Garmin Dakota 20, Garmin eTrex 20 and Garmin Oregon 200 GPS devices. Due to the geographical dispersal of the individuals, each animal was radio tracked approximately one night a week. If the animal was located inside an inaccessible garden, we recorded the location as being on the pavement just outside the garden. We continued radio tracking once a week during the hibernation period, and also recorded the number of nest changes during this period. Additionally, we monitored the weather conditions during the study period by extracting data from the Danish Meteorological Institute to investigate the effects of local climatic conditions on the behaviour of the hedgehogs, especially the timing of hibernation.

We radiotracked juvenile hedgehogs in seven western suburbs of Copenhagen and the more provincial town of Havdrup (Figure 1).

Figure 1. Map of study locations.

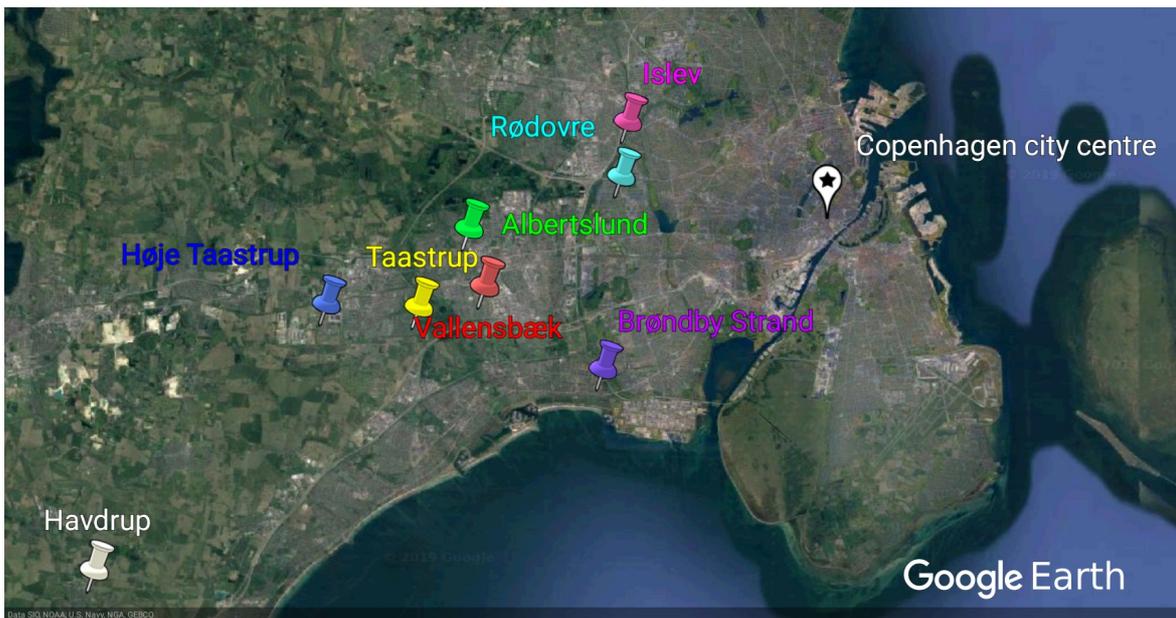


Figure 1. A map of Zealand, Denmark showing the locations of the radio tracked hedgehogs. Seven western suburbs were chosen (Islev, Rødovre, Albertslund, Taastrup, Høje Taastrup, Brøndby Strand and Vallensbæk), as well as the provincial town of Havdrup.

We estimated home range sizes for individuals for which we had at least 30 location points both before and after hibernation (Seaman et al. 1999). To do this we used the package `adehabitatHR` version 0.4.16 (Calenge 2006) in R (R Core Team 2019) to calculate the 50 % and 95 % minimum convex polygons (MCP) and 50 % and 95 % kernel density estimates (KDE) for each individual in both autumn and spring/summer. We thus calculated four estimates of home range area (in ha) per hedgehog, per season, when possible.

To investigate whether home range size varied between seasons and sexes, we fitted generalized linear models (GLMs) in R (R Core Team 2019) with a Gamma error structure, to account for non-normality. We used an inverse link function for the 95 % MCP and a logit link function for the 95 % KDE, 50 % MCP and 50 % KDE. Our response variable was home range size in hectares, and the explanatory variables were sex (female/male) and season (autumn/spring). We first fitted a maximal model including both explanatory variables and the two-way interaction between them (sex, season, sex:season). We then removed non-significant terms, interaction term first, and starting with the least significant, until we obtained a minimal adequate model where all remaining terms were significant (Crawley 2013). We tested significance of term deletions with by comparing AIC scores of the models, favouring the model with the lowest AIC.

Mortality and cause of death

In addition to tracking position, we collected data on mortality events in the hedgehogs we were following. If the cause of death was unclear, the hedgehogs were necropsied at Wildlifehealth.dk to clarify this information. Based on the mortality data we calculated Kaplan-Meier survival rates for all individuals combined and for both sexes, respectively. We used the Log-rank (Mantel-Cox) test to test for differences between the survival rates.

Weight and nutritional status

To monitor the health and development of the juvenile hedgehogs, we registered their nutritional status and weight. After the initial weighing and radio tagging, we weighed the hedgehogs as close to the onset of hibernation as possible. Post hibernation, the hedgehogs were caught and weighed at the first opportunity. Some hedgehogs were furthermore weighed during the autumn and spring. Due to challenges predicting the hibernation onset for each individual, and thereby obtaining a weight measure just before the onset of hibernation, we created linear regressions based on weight development during autumn for individuals with ≥ 2 recorded weights during the autumn, to estimate the weight of the hedgehogs on the exact dates of hibernation onset. These measures are hereafter referred to as the “estimated weight”.

We used the Bunnell Index (BI) (Bunnell 2002) as a measure of nutritional status. This index is calculated as the ratio of the circumference of the curled-up hedgehogs crosswise (A) and lengthwise (B) (i.e. A divided by B). A BI of > 0.8 is associated with a healthy animal with a satisfactory weight/size ratio.

Hibernation and nest changes

We radio tracked the hedgehogs once a week during hibernation, registering the nest changes and nest types used during hibernation.

Protection of animals in research

Our research was carried out in accordance with Danish Law (The Administrative Order on the Protection of Species, Artsfredningsbekendtgørelsen) and a permit to radio tag hedgehogs was granted by the Danish Nature Agency in September 2014 (J. Nr. SNS-41500-00210).

Results

Home range size

It was possible to calculate 22 home range areas using 95 % and 50 % minimum convex polygons (MCP) and 95 % and 50 % kernel density estimates (KDE) based on ≥ 30 GPS coordinates per individual during the autumn of 2014 and/or the spring/summer of 2015 (Table 1).

Table 1. Mean home ranges.

Mean home ranges for:	50% MCP	95% MCP	50% KDE	95% KDE	n
All individuals, all seasons	0.67	2.55	2.05	8.81	22
SD	0.85	3.21	2.81	11.01	
Autumn, all individuals	0.45	1.36	1.20	5.13	15
SD	0.57	0.75	1.28	5.03	
Spring/summer, all individuals	1.15	5.10	3.86	16.70	7
SD	1.19	4.85	4.25	16.08	
Autumn, males	0.32	1.33	0.89	4.15	9
SD	0.26	0.55	0.39	1.51	
Spring/summer, males	1.54	6.54	5.14	21.83	5
SD	1.20	5.10	4.47	16.49	
Autumn, females	0.65	1.40	1.66	6.60	6
SD	0.84	1.04	1.98	7.93	
Spring/summer, females	0.16	1.51	0.66	3.87	2
SD	0.03	0.88	0.15	1.71	

Table 1. Mean home range sizes measured in hectares.

The mean 95 % MCP home range area for males in the autumn was 1.33 ± 0.55 ha (mean \pm SD, n = 9) and 6.54 ± 5.10 ha (mean \pm SD, n = 5) in the spring/summer. Females had a mean 95 % MCP home range of 1.40 ± 1.04 ha during autumn (mean \pm SD, n = 6) and 1.51 ± 0.88 ha during spring/summer (mean \pm SD, n = 2). The mean 95 % MCP home range areas for both sexes combined in the autumn was 1.36 ± 0.75 ha (mean \pm SD, n = 15) and 5.1 ± 4.85 ha (mean \pm SD, n = 7) in the spring/summer. The mean 95 % KDE home range areas for both sexes combined in the autumn was 5.13 ± 5.03 ha (mean \pm SD, n = 15) and 16.7 ± 16.08 (mean \pm SD, n = 7) in the spring/summer (Table 1). One female individual had a home range of 0.43 ha during the autumn of 2014, covering only five gardens.

The minimal adequate GLM to describe the home ranges measured as 95 % MCP only contained season as an explanatory variable (AIC = 72.60, p = 0.001, residual deviance 10.16 on 18 df),

indicating that home ranges in spring were larger than in autumn. The next best model was the one containing season and sex as main effects (AIC = 73.258, Δ AIC = 0.66), then a model with the interaction between season and sex (AIC = 74.91, Δ AIC = 2.31) and finally the model only containing sex as a main effect (AIC = 86.42, Δ AIC = 13.82).

The minimal adequate GLMs to explain the home ranges measured as 50 % MCP, 50 % KDE and 95 % KDE all contained an interaction between season and sex ($y \sim \text{Sex} + \text{Season} + \text{Sex}:\text{Season}$). The next best model only had sex and season as main effects ($y \sim \text{Sex} + \text{Season}$), and were rejected due to a higher AIC-value. All the minimal adequate models for 50 % MCP, 50 % KDE and 95 % KDE showed that the autumn home ranges of the females were significantly larger than those of the males, and that the spring home ranges of the males were significantly larger than those of the females ((50 % MCP: AIC = 25.48, Δ AIC = 4.55, $p = 0.005$, residual deviance of 23.01 on 18 df); (50 % KDE: AIC = 67.75, Δ AIC = 6.26, $p = 0.004$, residual deviance of 11.96 on 18 df); (95 % KDE: AIC = 131.94, Δ AIC = 5.66, $p = 0.010$, residual deviance of 10.52 on 18df)) (Figure 2).

Figure 2. Home range plots.

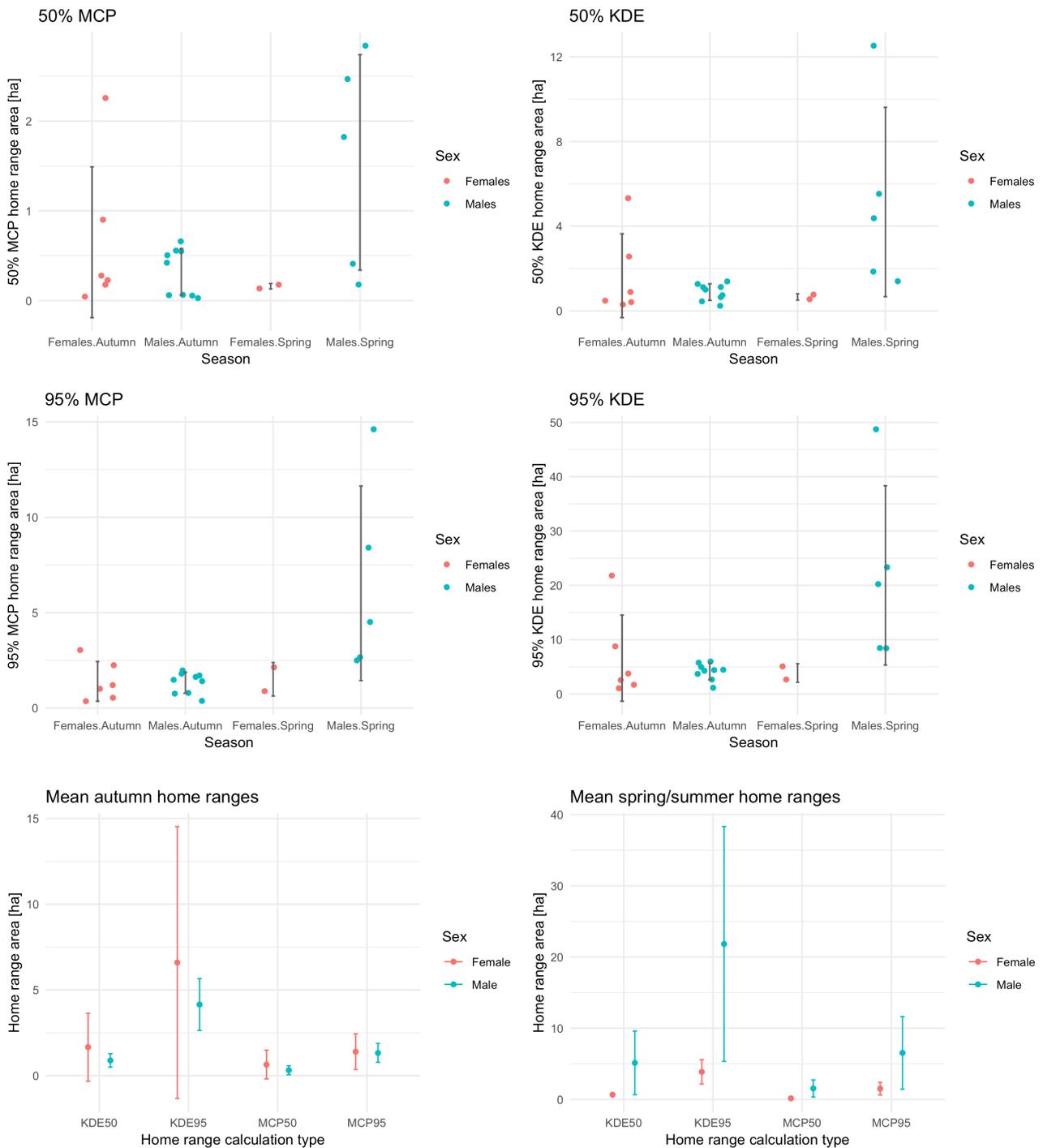


Figure 2. The first four plots: The home range sizes of the 22 calculated home ranges (of individuals with ≥ 30 recorded GPS locations), as 50 % MCP, 95 % MCP, 50 % KDE and 95 % KDE. The error bars show the standard deviation from the mean. The last two plots: Illustrating the differences in mean home range areas based on calculation methods, for males and females with error bars representing the standard deviation from the mean during the autumn of 2014 and spring/summer of 2015.

The young males in Taastrup (Figure 1) expanded their home ranges during the mating season to include novel areas. Individuals visited on average 10 gardens during the autumn of 2014 (range = 2-

20, n = 23), though not necessarily each night. This average rose to 14 gardens during spring/summer of 2015 (range = 7-20, n = 10).

The two most distant location points recorded per individual during the autumn ranged between 79-908 m, with a mean of $285 \text{ m} \pm 197.7$ (mean \pm SD, n = 23). The distance ranged between 156-843 m in the spring/summer, with a mean of 388 ± 206.4 m (mean \pm SD, n = 1).

The radio signals were lost from seven individuals during the study out of which two individuals were unaccounted for just after their tagging, perhaps due to defective tags. The seven individuals could not be found again and the signals from the radio tags were never retrieved in spite of a thorough search effort within a radius of 5 km from where they were last spotted. Due to the shedding of juvenile spines into adult spines, six individuals lost their tags before hibernation, two during hibernation, and four after hibernation (Table 2). Fortunately, we occasionally managed to reattach the tags to the same individuals again, leaving the total loss of tags to twelve incidences. One individual had two radio tags reapplied in the months after hibernation using a total of three tags in the study period. Several individuals had their glue reinforced or the tag moved and reattached whenever it was apparent that the tag became more and more detached and flabby due to the gradual shedding of spines. When retrieved, the detached tags were in good condition, with all the glue intact and a large number of spines attached to the glue. Due to the loss of tags before and during hibernation, eight individuals were excluded from the hibernation study. One individual was however seen alive in July 2015 and therefore counts as a survivor in the study. Four individuals lost their tags after hibernation. These individuals were never caught and radio tagged again in spite of a thorough search effort.

Mortality and cause of death

Nine of the 35 tagged individuals died during the study, of which two died during hibernation. Altogether, 17 out of 23 survived with certainty until the onset of hibernation. Two individuals died during hibernation. Seven individuals were known to have survived from their awakening from hibernation in late April or mid-May until the end of July. Unfortunately, 12 individuals lost their tags (six before hibernation, two during hibernation and four after hibernation), and seven individuals were unaccounted for due to lost radio signals (three before hibernation and four after hibernation) (Table 2).

Table 2. Overview of individuals.

	Before hibernation	During hibernation	After hibernation
Individuals tagged	33	0	2
Signals lost	3	0	4
Tags lost	6	2	4
Individuals dying	6	2	1
Individuals surviving	18/23	16/18	7/8
Percentage survival	78	89	88

Table 2. An overview of the number of dead individuals, the number of lost signals and tags (the individuals unaccounted for) before, during and after hibernation. The survival rates are only based on individuals that could be accounted for in the study.

The Kaplan-Meier survival probabilities for the period of September 2014-July 2015 were 0.70 for all individuals combined and 0.56 for females and 0.79 for males, with 26 cases of censored data (12 lost tags, seven lost signals, seven individuals surviving until the end of the study, Figure 3). The Log-rank (Mantel-Cox) test showed no significant difference between the survival curves (Chi square = 1.286, df = 2, p = 0.5257).

Figure 3. Kaplan-Meier survival plot.

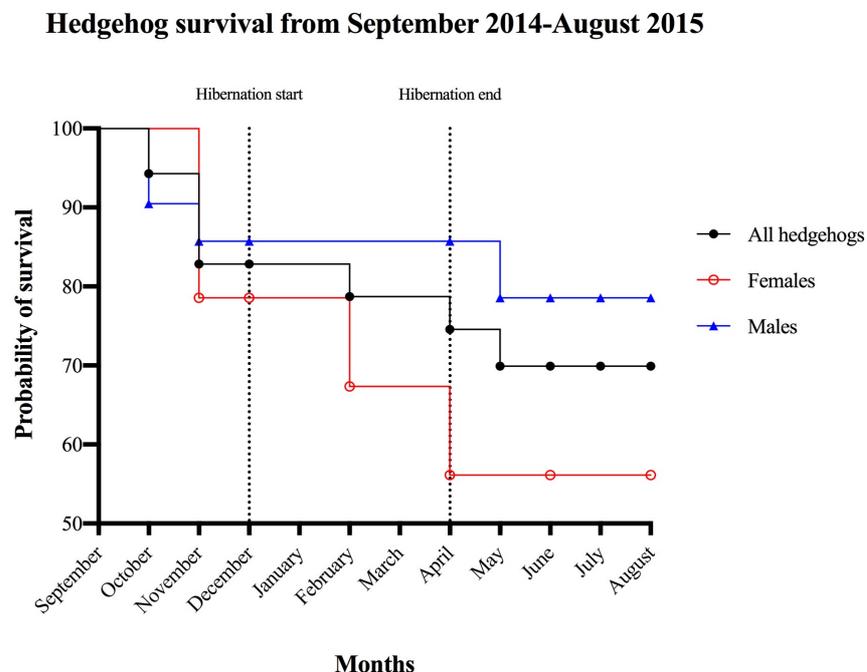


Figure 3. Kaplan-Meier survival curve representing the total number of juvenile hedgehogs (n = 35, black) in the study as well as the females (n = 14, red) and males (n = 21, blue). 26 data points were censored, due to unknown fate after the study ended (n = 7), loss of radio tag (n = 12) and loss of radio signal (n = 7). The calculated survival proportions with censored data was 70 % for all hedgehogs, 56 % for females and 79 % for males. The Log-rank (Mantel-Cox) test showed no significant difference between the survival curves (Chi square = 1.286, df = 2, p = 0.5257).

The causes of death in these individuals varied. One individual was euthanized at Copenhagen Animal Hospital due to a self-inflicted, lethal injury, as it was entangled in thorn branches, and another was taken into care by a hedgehog rehabilitator but did not survive. Necropsies of two individuals, performed at Wildlifehealth.dk, revealed that they died from *Salmonella* infections: one during the autumn and one during hibernation. Both visited the same feeding station, from where they likely contracted the infection. Green, watery and smelly faeces was observed near the food bowl visited by several hedgehogs including the two radio-tagged individuals during the autumn of 2014. One individual drowned in an artificial stream surrounded by high concrete walls and another may have died from poisoning with rodenticides but was not tested. Two individuals died due to fox attacks (one during hibernation) and one due to either a dog or fox attack. The last cause of death was shredding with garden waste, as the day nest was situated inside a pile of branches, which was destroyed in a garden waste shredder.

Weight and nutritional status

All individuals gained weight during autumn, except for two individuals dying from *Salmonella* infections. Healthy individuals weighing from 213 grams in September reached a weight of up to 700 grams prior to hibernation.

Some individuals took a large effort to catch both before and after hibernation, causing their before and after weights to be somewhat unrepresentative of the actual weight loss during hibernation (e.g. individuals 17 and 29, Table 3). However, we measured the weight development during hibernation with the available data and estimated the weight development based on the exact date of hibernation by use of regression lines (Table 3).

Table 3. Weight development.

Individual	Weight before hibernation				Weight after hibernation							Nest changes
	Hibernation start	Date of weighing	Weight (g)	Estimated weight at hibernation onset (g)	Hibernation end (2015)	Date of weighing (2015)	Weight (g)	Weight change (g)	Estimated weight change (g)	% weight development	% estimated weight development	
1	19/11/14	2/11/14	505	624								0
2	21/11/14	29/10/14	570	801	2/5/15	13/5/15	653	83	-148	15%	-18%	1
7	16/11/14	29/10/14	755	924	24/4/15	14/5/15	665	-90	-259	-12%	-28%	0
8	19/11/14	1/11/14	748	947	24/4/15	24/4/15	577	-171	-370	-23%	-39%	8
14	11/11/14	16/11/14	504									1
16	28/11/14	15/11/14	551	676	29/4/15							0
17	5/12/14	20/10/14	470		9/5/15	19/5/15	754	284		60%		0
18	30/11/14	16/11/14	480	574	2/5/15	19/5/15	645	165	71	34%	12%	0
20	21/11/14	11/11/14	586	657	29/4/15							3
23	30/11/14	15/10/14	424		14/4/15							3
25	30/11/14	22/11/14	525	574	14/4/15	21/4/15	483	-42	-91	-8%	-16%	0
27		30/10/14	373									Lost tag
28	2/1/15	6/1/15	472	379	14/5/15	14/5/15	446	-26	67	-6%	18%	0
29	5/12/14	29/10/14	488		24/4/15	19/5/15	752	264		54%		1
30	2/12/14	11/11/14	553									Lost tag
31	8/12/14	17/11/14	656	807	30/4/15	17/5/15	749	93	-58	14%	-7%	0
32	8/12/14	2/11/14	589	1148	30/4/15	30/4/15	590	1	-558	0%	-49%	1

Table 3. The hibernation and weight data (in gram) of the hedgehogs in the study. Individuals 1 and 14 died during hibernation. Individual no. 23 was killed a few days after hibernation and was therefore not weighed after hibernation. Individuals 16, 20, 27 and 30 lost their tags during hibernation or when waking up and leaving the nest, which made it impossible to obtain their post hibernation weights. Individual 28 hibernated in a nest box at a hedgehog rehabilitation center (0 nest changes). The calculated weight is based on linear regression (for individuals with ≥ 2 weight records during autumn) in order to provide an estimate for the weight on the exact date of hibernation onset.

Of the ten individuals with sufficient data to represent the weight development during hibernation, four lost weight, five gained weight and one had no weight development (mean = +13 % \pm 2.8 SE). It was possible to calculate the estimated weight development from the date of hibernation onset for eight individuals, out of which six lost weight and two gained weight (mean = -16 % \pm 2.9 SE). The individuals losing weight during hibernation e.g. 12 % weight loss from 755g to 665g (estimated weight loss of 28 %), were in very good condition before hibernation. One individual lost 23 % weight during hibernation (estimated weight loss of 39 %), which was the highest recorded weight loss of this study. This individual made eight nest changes during the hibernation period, and perhaps even

more unrecorded, and was active almost weekly throughout the winter. The individual was still in good condition after awakening from hibernation, weighing 23 % less (577g). Very few individuals, only the two described earlier, had a documented weight loss of more than 50 grams during hibernation. The autumn and winter weather during this study was exceptionally mild (Danish Meteorological Institute 2014; Danish Meteorological Institute 2015b), leaving the hedgehogs in excellent condition before hibernation and hence in good condition after hibernating.

The female hedgehogs gained weight throughout the spring/summer. One male kept a relatively steady weight in spite of a large expansion of home range during the mating season. However, two males weighed from mid-May and onwards lost weight during the mating season.

Nutritional status, as estimated by the Bunnell Index (BI), varied markedly among animals with some being fairly healthy and others emaciated. We calculated 36 BIs for a total of 21 individuals and the overall mean BI was 0.82 (range 0.71 to 0.90). One individual had a BI of 0.74 when found in a severely emaciated state, dying from *Salmonella* infections on the 2nd of November 2014. However, the individual with the lowest BI of 0.71, weighing 748 grams on the 19th of May 2015 was in excellent condition.

Hibernation and nest changes

In 2014, the first night frost was on November 30th (Danish Meteorological Institute 2014) and we recorded the first individual (out of 18) to enter hibernation on November 16th weighing >700 grams. Six individuals began hibernation between November 16th-21st, four individuals went into hibernation between November 28th- 30th and five individuals waited until December 2nd- 8th. The last tagged individual began hibernation on January 2nd 2015 at a wildlife rehabilitation centre.

The number of nest changes recorded per individual ($n = 15$) during hibernation ranged between 0 and 8, with most individuals changing nest 0-1 times (median = 0, range = 0-8, mean = 1.2 ± 0.14 SE). Most individuals stayed in one garden during hibernation but others ranged more widely: the individual with eight recorded nest changes visited at least 10 gardens during the hibernation period. The animals resumed post hibernation activity between April 4th and May 14th 2015 ($n = 13$).

Discussion

Our results showed relatively small home range sizes and a high survival probability of radio tracked individuals living in a suburban habitat, compared to previous studies of juvenile hedgehogs. In the following section, we will compare the results to past research, and discuss the application of the Bunnell Index as a measure of hedgehog health, as well as the effects of local climatic conditions on the ecology of the hedgehogs. We will furthermore consider the challenges of radio tagging juvenile

hedgehogs and radio tracking in a suburban habitat and provide suggestions for improvements of this type of research in the future.

Home range size

The home ranges calculated in this study (95 % MCP: mean autumn 1.36 ± 0.75 ha (mean \pm SD, $n = 15$) and mean spring/summer 5.1 ± 4.85 ha (mean \pm SD, $n = 7$)) (Table 1) were generally small in comparison with home ranges from previous studies from Scandinavia, which have reported home range sizes of 2.6-98 ha (adults), which were however measured with different methods (Kristiansson 1984b; Rautio et al. 2013; Riber 2006; Sæther 1997). This could indicate that the suburban environment, at least of this study, possesses great food resources for the hedgehogs enabling them to save energy on foraging and keeping smaller home ranges. We also observed that individuals tended to stay in the vicinity of the local feeding station with cat food and water, moving around in gardens surrounding this particular garden, especially during the autumn of 2014, as was the case with an individual only residing in approximately 4-5 gardens during the autumn (95 % MCP home range of 0.43 ha). We did not regard the feeding stations as confounding factors in our study, since we expected that some garden owners in a residential area would feed the hedgehogs (or their cats) in the garden and therefore it would be a common situation in a suburban habitat (Morris 1985).

We found a statistically significant effect of sex on the home ranges sizes, with home ranges of females being larger than those of males in the autumn, and home ranges of males being larger than those of females during the spring/summer (for calculated 50 % MCP, 50 % KDE, 95 % KDE). Using 95% MCP we found no effect of sex on the home range areas, but the home ranges were generally larger during spring/summer (95 % MCP). Earlier studies have shown that male hedgehogs tend to have larger home ranges than females, and this perception seems to have been generally accepted (Morris 2014). In a residential area near Trondheim, Norway, Sæther (1997) found no difference between the home ranges of male and female juveniles during the summer of 1995. However, the results of our study may have been affected by one particularly active female individual during the autumn of 2014, due to the low sample size (autumn: 6 females, 9 males). We did find that the home ranges of both males and females were larger during the mating season in spring, as expected. There was a tendency for the individuals in Taastrup to gather in the recreational spaces surrounding the inhabited areas during the mating season. They would nest in their normal area and move towards the open, recreational spaces and grain fields at nightfall.

Few of the hedgehogs dispersed more than 500 m from where they were radio tagged during the 10 months the study lasted, even though many of the individuals were caught in their birth area and were expected to disperse (Berthoud 1978; Morris 2014; Reeve 1994). Doncaster et al. (2001) did

suggest, however, that hedgehogs do not have a fixed natal territory from which to disperse, nor a clearly defined dispersal stage. Sæther (1997) found that male juveniles ($n = 17$), which had recently become independent, dispersed 230 m from the natal nest and females dispersed 223.5 m from the natal nest. We observed one incidence of parents chasing their own juvenile offspring away from the feeding station in their natal area, and this only took place *after* hibernation. The parents accepted the presence of their independent offspring during the autumn. In this particular case, the adults stayed in the same area and remained in a sort of cohabitation after the mating season. The male and female both lived in the same garden shed from September 2014 to June 2015. The male slept in a nest next to the nest inhabited by the female and her offspring. The adults would follow the juveniles around together from the time the juveniles would leave the nest (at 3-4 weeks of age) until they reached independence (at around 6-7 weeks of age).

A large number of tags (12 out of 35 attached) were lost possibly due to the shedding of spines. This is a high number compared to previous studies using the same tag types, glue and attachment techniques. The shedding of spines, as they are replaced by adult spines, is normally a gradual process, but perhaps the excellent food availability caused by the great climatic conditions in the autumn, made the hedgehogs develop their adult spines faster making the shedding more sudden than the normal gradual process? Perhaps the hedgehogs in the study could have become stuck under garden fences more often compared to individuals previously studied in more open landscapes with less of those features. Most shed tags were retrieved and had intact glue with several spines still attached. The spines had bulb roots at the end as seen in regular shedding processes. The loss of radio tags due to shedding reduced the number of individuals followed throughout the study, until the last night of fieldwork in July, to seven.

Apparently, no individuals were hit by cars during the study, but at least two with lost signals were known to cross relatively busy roads in the summer, where a number of hedgehogs are killed by cars every year. These roads are frequently visited by scavengers like foxes and gulls effectively removing road-kills. A signal was lost from an individual in the more rurally situated suburb of Havdrup, the only study site inhabited by badgers. Foxes were present in all the locations.

Survival

A high survival probability of juvenile hedgehogs during their first year of life is important for the growth and maintenance of the general hedgehog population. The survival probabilities for the hedgehogs in this study (Kaplan Meier: 0.7 for all individuals, 0.56 for females and 0.79 for males) were rather high compared to previous studies of juvenile hedgehogs in Scandinavia, which range between 0.31-0.66 (Kristiansson 1990; Rasmussen 2013; Sæther 1997). The survival probability of

0.89 (16 out of 18 individuals) during hibernation in this study is also quite high compared to the estimates of 0.66 and 0.69 by Kristiansson (1984b) and Walhovd (1990). Nevertheless, Jensen (2004) found a 100 % survival rate during hibernation for the six juveniles studied. Our findings may indicate that the juveniles have greater survival chances in a suburban habitat. However, further studies are needed before such a conclusion can be confirmed.

Weight development and nutritional status

Some individuals took a large effort to catch both before and after hibernation, causing their pre- and post-hibernation weights to be unrepresentative of the actual weight loss during hibernation. It was possible to calculate the expected weight on the date of hibernation onset for eight individuals, using regression lines. However, weight gain after hibernation followed by a weight loss for the males during the mating season made the weight development appear polynomial instead, making calculations on expected post hibernation weights uncertain. Comparing with previous studies of hibernation weight loss in Danish, juvenile hedgehogs, Jensen (2004) calculated a mean weight loss of 22.1 % \pm 10.1 (mean \pm SE) weight during hibernation (n = 10), the lightest individual only losing 4.5 % weight (23 grams). Rasmussen (2013) found that one individual went into hibernation weighing around 450 grams and had only lost 20 grams (4.5 %) when retrieved in May 2013. Our estimate of mean weight loss, calculated based on regression lines, was 16 % \pm 2.9 (mean \pm SE) for individuals with an estimated pre-hibernation weight of 379-1149 grams.

We used the Bunnell Index (Bunnell 2002) as a supplementary measure of nutritional status. This index indicates the condition of an animal with regard to nutritional status, because weight does not take skeletal size into account, and is therefore not necessarily a reliable measure of nutritional status. For example, a small hedgehog of 600 grams would be in good condition, while a large hedgehog of 600 grams would be in a poor condition. However, the results are completely dependent upon precise measures, which can be challenging in the field. Furthermore, the Bunnell Index also varies according the personality of the hedgehog, the degree of habituation to human contact and therefore the tendency for a hedgehog to curl up tightly or in a more relaxed manner. This proved a challenge especially when tracking weight developments of specific individuals that would behave differently in the first, second and third weighing. Therefore, we found that the Bunnell Index was a somewhat unreliable method for determining nutritional status of hedgehogs. Based on the weight development of the hedgehogs before and after hibernation, it appears that the individuals that could afford to lose most weight did in fact lose most weight, as was also seen in the study by Jensen (2004), Rasmussen (2013) and Morris & Warwick (1994).

Nest changes during hibernation

Nest changes do take place during the hibernation period, and we radio tracked the hedgehogs on a weekly basis during the winter of 2014/15. One individual changed nests eight times during hibernation and used 7 different nests. However, it is possible that the individual did in fact change nests more frequently in between the events of radio tracking. Jensen (2004) found that the average number of nest changes during hibernation was two, with a single individual changing nests four times. Morris (1973) found that only two nests out of 167 studied remained occupied for the whole winter, and all others were vacated as the animals moved to another nest. Except for one individual, we found that the hedgehogs only changed nests one or three times, and several individuals did not change nests at all. However, if an individual had left the nest to forage and went back into the nest again, the activity would only have been recorded if the individual was out of the nest in the instant it was radio tracked. Nest change as a measure of activity during hibernation is somewhat difficult to detect and quantify accurately, which is why the use of accelerometers for future studies of hibernation would be a preferable method.

The influence of climate

Our findings indicate that the mild climatic conditions in the autumn of 2014 influenced the hibernation behaviour of the radio tracked hedgehogs, the food availability, and hence the weight development and survival as well as the breeding pattern of adult hedgehogs. The Danish autumn of 2014 was the second warmest since 1874, with an average temperature of 11.9 °C. There were only 1.4 frost days in the period, which is exceptionally low (10 days on average). The autumn's first temperatures below 0 °C occurred during the night on the 30th of November. The mild autumn weather caused food resources like slugs and snails to be available at least until the 1st of December, which is unusual in Denmark. The Danish winter of 2014/15 was the 9th warmest winter since 1874, with an average temperature of 2.8 °C and 27.2 frost days. The winter was however the 7th wettest winter since 1874 with a rainfall of 245 mm (Danish Meteorological Institute 2015b). The mild Danish autumn and winter of 2014-15 undoubtedly affected the survival of the juvenile hedgehogs in the study by decreasing mortality. Furthermore, we recorded two adult females giving birth to second litters during the mild autumn of 2014, which is the first record of its kind in Denmark. As an example, individuals no. 34 and 35 were born in the first litter and no. 23, 24 and 25 came from the second litter of a female hedgehog residing in a garden shed in Rødovre.

Compared to previous observations from Denmark (Jensen 2004; Rasmussen 2013; Walhovd 1976; Walhovd 1978; Walhovd 1990), the juveniles in this study entered hibernation much later. This is likely linked to the extremely mild weather conditions during the autumn, 2014 with the first night

frost as late as November 30th, which is late for a Danish standard (Danish Meteorological Institute 2014). As a consequence of the mild November temperatures, food resources such as slugs and snails were even available in the beginning of December.

Phenology studies of UK hedgehogs have failed to detect an association between climate conditions and the timings of post-hibernation emergence of hedgehogs (PTES & BHPS 2015). As observed in previous studies (Jensen 2004; Rasmussen 2013; Walhovd 1978), we also found that hedgehogs resumed activity after hibernation between mid-April and mid-May, after a winter with average climatic conditions (Danish Meteorological Institute 2015b). However, our findings indicate that the start of hibernation may be influenced by local climatic conditions due to its effect on the availability of food resources. These results were subsequently confirmed by observations of delayed hibernation start and excellent weight gain in Danish, juvenile hedgehogs during the mild autumns of 2015 and 2018 (Danish Animal Welfare Society 2018; Danish Meteorological Institute 2015a; Danish Meteorological Institute 2018).

Radio tracking in a suburban habitat

Inferences from radio tracking data depend on the frequency and precision of the radio tracking. Fieldwork in this study took place almost every night in the autumn of 2014 and spring/summer of 2015. However, due to the wide distribution of study locations, we necessarily focussed on a single suburb per night, meaning that each individual was on average only followed one night per week. We therefore acknowledge that our tracking data likely do not represent the full extent of individual movement patterns and we may consistently underestimate home range size.

The suburban habitat of the hedgehogs proved to be a challenging environment for radio tracking. There was rarely visual contact whenever the hedgehogs moved around from garden to garden with high fences or hedges. Most location points were registered outside the actual garden in which the hedgehog would be present, making it impossible to observe their behaviour. Constant access was granted to a number of gardens, especially the gardens with feeding stations, where most of the hedgehogs in the study were caught. The hedgehogs regularly visited the food bowls and this also turned out to be the easiest way to recapture the individuals for the weighing before and after hibernation.

Suggestions for future hedgehog studies

GPS tags can include built-in radio tags and accelerometers and therefore provide more consistent data of spatial behaviour and activity levels, providing information on the effect and importance of garden connectivity on hedgehogs and enabling researchers to obtain a larger sample size covering a larger geographical area, without disturbing and influencing the behaviour of the hedgehogs studied

(Barthel et al. 2019). Furthermore, the GPS tags could provide detailed information about the movement barriers affecting the hedgehogs in suburban areas. However, urban barriers may also in special cases deflect GPS signals.

Due to the intensified agriculture, with larger fields, the loss of hedgerows and grassland and use of pesticides, it is likely more beneficial to direct future conservation initiatives at the improvement and adaptation of urban and suburban habitats for hedgehogs to stop the drastic population decline seen in the UK (SoBH 2011). It would therefore perhaps be more constructive to focus the research into hedgehog ecology on urban habitats, investigating which anthropogenic dangers that influence the survival of hedgehogs, how to reduce mortality rates and thereby target our conservation strategies towards hedgehogs in the most efficient way possible.

Conclusion

We radio tracked 35 juvenile hedgehogs in residential suburbs of western Copenhagen from 20th September 2014- 22nd July 2015. Nine individuals died, 12 lost their tags, and the signal was lost from seven individuals. The survival probability was high compared to previous research from Scandinavia. It is possible that the mild climatic conditions during the autumn of 2014 made food items abundant, causing the individuals to gain weight fast and, in some instances, even delaying the onset of hibernation, which was considerably later than recorded in previous studies from Scandinavia. We furthermore recorded two incidences of second litters due to the favourable climatic conditions. Only two hedgehogs did not survive hibernation. Few hedgehogs seemed to lose more than 50 grams during hibernation, and the heaviest hedgehogs lost most weight. We found that home ranges became larger during the spring and summer of 2015 compared to the autumn of 2014, which was expected due to the onset of the mating season. The home ranges of this study were generally smaller than those found in past studies from the UK, Norway, Sweden and Denmark. However, previous research has not focused on juvenile hedgehogs in suburban areas.

Our study has contributed with knowledge on the ecology of juvenile hedgehogs in suburban habitats and the impact of local climatic conditions on the behaviour of the hedgehogs in the period of September 2014-May 2015 in a Danish setting. However, further studies representing more time series and locations are needed to provide the sufficient knowledge and data about hedgehog ecology in urban habitats to improve the conservation strategies in the area, and draw solid conclusions on the effects of climate change on hedgehog behaviour.

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Manuscript II

Genetic structure of the European hedgehog (*Erinaceus europaeus*) in Denmark



Genetic structure of the European hedgehog (*Erinaceus europaeus*) in Denmark

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Abstract

We aimed to determine the genetic structure of a nationwide sample of European hedgehogs (*Erinaceus europaeus*) from Denmark by adapting the genotyping by sequencing (GBS) technique to the study of hedgehogs. We analysed 178 individuals from six geographically and genetically distinct populations.

We estimated inbreeding coefficients (F), observed (H_O), expected (H_E), and unbiased expected (uH_E) heterozygosity and the percentage of polymorphic loci ($P\%$). We tested for deviations from Hardy-Weinberg equilibrium (HWE) and patterns of isolation by distance (IBD). We determined the genetic structure of the sampled individuals based on a Bayesian clustering method, and tested for recent population increase or decline. The Danish hedgehog population was divided into three clusters.

We found a mean $P\%$ of 54.44-94.71, a mean uH_E of 0.126-0.318 and a mean H_E of 0.121-0.316 and a mean H_O of 0.124-0.293 in the six populations. The heterozygosity deficiency was reflected in positive F values for five populations and a significant deviation from HWE in three of six populations tested ($p < 0.05$). The Mantel test for association between genetical and geographical distance within populations was non-significant (range $b = 0.006$ - 0.08 , $R^2 = 0.029$ - 0.046 , $0.062 < p < 0.1$). We tested for effects of densities of human populations, roads and farmland on the genetic variability and found that F decreased with increasing farmland density ($t = 3.099$, $p = 0.0363$, $df = 4$).

The significant and positive inbreeding coefficients found in the majority of the Danish hedgehogs tested, can be explained by inbreeding, genetic substructure and low effective population size, which are all consequences of habitat fragmentation. We failed to detect signs of a recent population bottleneck or population increase or decline. However, as the available test only identifies very recent changes in population size, we cannot reject the possibility of a longer-term decline in the Danish hedgehog population.

Introduction

The western European hedgehog (*Erinaceus europaeus*, hereafter referred to as “hedgehog”), is found on the British Isles, New Zealand and Continental Europe, from Iberia and Italy northwards into Scandinavia. The species is generally common and widely distributed, and can survive across a wide range of habitat types (Morris 2014; Reeve 1994). Hedgehogs are nocturnal and non-territorial generalist predators and can travel up to 2-3 km a night. Home ranges of adult hedgehogs are generally estimated to 20-30 ha for males and 10 ha for females, expanding temporarily during the mating season (Morris 2014). Juvenile hedgehogs do not disperse far when reaching independence and leaving their natal nests (Sæther 1997) and adults appear to remain in the same area throughout their lives (Reeve 1994). In addition, studies on relocated hedgehogs indicate that they do not disperse far even when released into a foreign (Rasmussen 2013) and unfavourable habitat (Doncaster et al. 2001).

Previous research on both national and local scale from several western European countries, reports decline, or concerns for decline, in hedgehog populations in the UK, Belgium, the Netherlands, Sweden and Germany (Hof & Bright 2016; Holsbeek et al. 1999; Huijser & Bergers 2000; Krangle 2015; Müller 2018; SoBH 2011; SoBH 2015; SoBH 2018; van de Poel et al. 2015; Williams et al. 2018). Although there is currently a lack of data to indicate the status of the Danish population it is likely that the situation is similar because of comparable habitat fragmentation, landscape structure, farm management practices and climate across north-western European countries.

The potential drivers of the decline include habitat loss, which has caused and continues to cause severe species loss in terrestrial ecosystems in general (Brooks et al. 2002). Intensified agricultural practices often include removal of hedgerows to create large homogenous and exhaustively managed fields (Stoate et al. 2001). Hedgerows and other edge habitats are important habitats for hedgehogs living in rural areas (Hof & Bright 2010; Riber 2006). The removal of edge habitats from arable fields also limits connectivity between suitable habitats (Michel et al. 2006). Intensified agricultural practices lead to reduced habitat quality for hedgehogs and in the UK it is suggested that the hedgehog decline is more severe in the rural than urban areas (SoBH 2018; Williams et al. 2018).

Widespread application of molluscicides, insecticides and rodenticides also has a negative effect on hedgehog populations via secondary poisoning and elimination of prey items (Brakes & Smith 2005; Dowding et al. 2010b; Haigh et al. 2012; Robinson & Sutherland 2002). Environmental pollution in general may also affect hedgehogs (Rautio et al. 2010). Badgers

are intra-guild predators of hedgehogs, but can moreover negatively affect hedgehog survival by competition for food resources and habitats (Hubert et al. 2011; Micol et al. 1994; Pettett et al. 2017; van de Poel et al. 2015; Young et al. 2006). Previous research indicates that hedgehogs are increasingly associated with residential areas, possibly due to greater food availability around humans (including natural prey and anthropogenic sources), more suitable nest sites and a lower risk of predation by badgers (*Meles meles*) (Doncaster et al. 2001; Hubert et al. 2011; Micol et al. 1994; Morris 1985; Pettett et al. 2017; van de Poel et al. 2015; Young et al. 2006).

The fragmentation of the landscape by roads is a source of mortality through roadkill, as well as a barrier to movement for the hedgehogs (Dowding et al. 2010a; Huijser & Bergers 2000; Rautio et al. 2016; Rondinini & Doncaster 2002). Habitat connectivity is important for the movement and dispersal of animal species such as the hedgehog due to the enhanced gene flow, with potential benefits for fitness, adaptability and survival of local populations (Braaker 2017; Reed 2004). Habitat fragmentation is considered most pronounced in cities (Grimm et al. 2008), but for species such as the hedgehog, green spaces within the cities can form an important network of habitats (Hof & Bright 2009).

Hedgehogs are promiscuous and have heteropaternal superfecundation (Moran et al. 2009), which may have a positive effect on inbreeding, since a litter can consist of several half-siblings instead of full siblings (Moran et al. 2009). It is currently unknown whether hedgehogs are able to actively differentiate between kin and non-kin e.g. during the mating season, but if a small, isolated population is severely inbred, and there is no distinction between kin and non-kin when choosing mates, the population will most likely become even more vulnerable due to the increased degree of inbreeding.

Genetic diversity of hedgehogs

Reduction of suitable habitats and loss of genetic diversity are believed to be the major influential factors leading to the extinction of wildlife populations (Keller & Waller 2002; Lacy 1997). Maintaining genetic connectivity across fragmented landscapes is necessary to prevent the negative consequences of genetic drift and inbreeding. The tools of conservation genetics, including genetic rescues, are useful for assessing, conserving and managing genetically vulnerable, fragmented populations (Ralls et al. 2018). Research into the hedgehog population at a molecular level is therefore relevant for quantifying the conservation status of a hedgehog population.

Previous genetic research on hedgehogs has been conducted using microsatellites (Barthel 2019; Becher & Griffiths 1997; Becher & Griffiths 1998; Berggren et al. 2005; Bolfikova & Hulva 2012; Bolfikova et al. 2013; Bolfikova et al. 2017; Braaker 2017; Fraser et al. 2012; Henderson et al. 2000; Moran et al. 2009; O'Reilly 2016; Santucci et al. 1998; Seddon et al. 2001). Bolfikova & Hulva (2012) used population and landscape genetic approaches to describe the population structure and patterns of gene flow of *E. europaeus* and *E. roumanicus* in the central European contact zone between the two species, and found that a homogenous population of *E. europaeus*, had been divided by two large rivers in the Czech Republic (Vltava and Elbe), into a subpopulation in the western part, and two subpopulations with a mosaic location pattern in the eastern part of the country. They found a significantly lower observed heterozygosity (H_O) than expected heterozygosity (H_E) in five of the nine microsatellite loci studied ($n = 131$), using the mitochondrial control region and nuclear microsatellites. In the UK, a study of 42 individuals in an isolated population of hedgehogs in the Regent's Park, London (166 ha), showed a low genetic diversity, with a mean H_E of 0.197, and a mean H_O of 0.198 ($n_{\text{loci}} = 6$) (O'Reilly 2016). Becher & Griffiths (1998) detected a restriction of gene flow between eight small populations of hedgehogs in a 15 km² fragmented landscape in Oxfordshire, UK and found a statistically significant genetic differentiation among the studied populations ($n = 160$, $n_{\text{loci}} = 6$) and a mean H_O of 0.7. These results indicate that the hedgehogs of Oxfordshire had a restricted dispersal which may have been caused by human-mediated barriers such as roads and train tracks in the landscape (Becher & Griffiths 1998). Braaker (2017) studied the habitat connectivity and spatial genetic structure of 147 hedgehogs residing in Zurich, Switzerland, with an area of 88 km² ($n_{\text{loci}} = 10$). The population of hedgehogs in Zurich were divided into three genetic clusters, separated by two rivers and the major transportation axes. Genetic diversity measures were similar between the three clusters, and the inbreeding coefficients were low. Mean H_E ranged between 0.569-0.627 for the three clusters, and the mean H_O ranged between 0.523-0.631 (Braaker 2017).

The Danish context

The earliest recordings of hedgehogs in Denmark date to approximately 7500 BC, found in archaeological excavations at four Mesolithic sites (Maglemosian cultures), even though they are thought to have immigrated already in the Early Preboreal around 9550 BC (Aaris-Sorensen 2009).

Denmark consists of the large peninsula Jutland and several islands of varying sizes. The larger islands are connected by long bridges (0.75-17 km), which hedgehogs are unlikely to

cross, isolating the local hedgehog populations in the different areas of Denmark. The total area of Denmark is 43,000 km² (Statistics Denmark 2019a), and almost a third of this, 62 %, is arable land (Statistics Denmark 2017). Altogether, Denmark has 74,728 km of roads and 1,737,000 cars for 5,800,000 people (Statistics Denmark 2018; Statistics Denmark 2019b; The Danish Road Directorate 2019). The number of cars in Denmark has increased with 22 % since 2008 (The Danish Road Directorate 2019), and an increase in road traffic could likely influence the number of hedgehogs being killed by cars (Elmeros 2014).

For this study the use of a second-generation genotyping technique, genotyping by sequencing (GBS), was adapted and optimised for the genetic analysis of European hedgehogs. The main aims of the experiment were: (1) To provide a set of SNPs which can be used for investigating the genetic structure and variability of the European hedgehog on a broader scale (2) to evaluate the patterns of the genetic diversity distribution in the Danish hedgehog populations and (3) to ascertain the historical changes in their effective population size (N_e) through genetic signatures.

Materials and Methods

The genetic samples were obtained as part of a nationwide citizen science project with the general aim of describing hedgehog ecology in Denmark. By use of local and national media and a project website, volunteers were encouraged to collect dead hedgehogs from May to December 2016. A total of 697 dead hedgehogs originating from all parts of Denmark, were collected. The volunteers were instructed to record the date and location of the find and deliver the dead hedgehog to the nearest of 26 collection stations, distributed nationally. The hedgehog carcasses were stored locally at -20 °C. Members of the research staff regularly transported the collected, dead hedgehogs to university laboratories, where they were thawed and necropsied from August 2016 to May 2018. During the necropsies, tissue samples from skin and muscle were obtained for the genetical analyses. The DNA samples were stored at -20 °C.

Genetic samples from 178 individuals dispersed throughout Denmark were used in the study (Table 1).

Table 1. Distribution of genetic samples in the study.

Population	Location	Area in km ²	Abbreviations	Number of individuals
1	Jutland north of the Limfjord	5340	JNL	9
2	Jutland south of the Limfjord	23873	JSL	71
3	Funen	3479	F	15
4	Zealand	7031	Z	51
5	Falster and Lolland	1797	LFA	18
6	Bornholm	588	BH	14
Total:				178

Table 1. An overview of the geographical distribution of genetic samples in the study.

Sample preparation

DNA was extracted from muscle and skin tissues (1-2 mg) using the DNeasy Blood and Tissue kit (QIAGEN, Germany) and subsequently digested with *Sau96I* (NEB) and ligated to adapters as described elsewhere (Elshire et al. 2011). The ligated samples (50µL containing approximately 50 ng DNA pooled from 4 separate samples) were purified with AMPure XP beads (Beckman Coulter, USA) and amplified using the Phusion High-Fidelity PCR kit (Thermo Scientific, USA). The following PCR conditions were applied: 72 °C in 5 min, 98 °C in 30 sec, followed by 20 cycles of 98 °C in 10 sec, 66 °C in 30 sec and 72 °C in 30 sec, with a final extension at 72 °C for 5 min. Finally, the amplified barcoded DNA were purified with AMPure XP beads (Beckman Coulter, USA) and the DNA concentrations were determined by Qubit (Thermo Scientific, USA) and visualized on the TapeStation 2200 using a D1000 ScreenTape (Agilent, USA). Paired-end (2x151 bp) sequencing was performed on an Illumina HiSeq X platform at Admera Health (South Plainfield, NJ, USA).

Data analysis

The i7 barcodes of the dual-barcoded sequenced reads were demultiplexed using bcl2fastq2 version 1.0.0 (Illumina, Inc., San Diego, CA, USA) allowing zero mismatch. The i5 barcodes were demultiplexed using Fastq-multx version 1.02.772 (<https://github.com/brwnj/fastq-multx>) allowing one mismatch in order to remove the barcode sequences from the sequenced reads.

Filtering raw sequence data, mapping and SNP calling

Adaptor quality trimming were accomplished by Trim Galore using default parameters (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore). Burrows-Wheeler Aligner

(BWA) was used to align the reads against the hedgehog (*Erinaceus europaeus*) using the EriEur2.26 reference genome. Only reads with a mapping quality of at least 30 were used for the further analysis. Variants were called using The Genome Analysis Tool Kit's HaplotypeCaller, and joint genotyping was performed using GenotypeGVCFs. Initial filtering was performed using SelectVariants and filtered for SNPs, bi-allelic sites, and mapping quality > 30 (DePristo et al. 2011). Minor allele frequency (MAF) was estimated from the read coverage, and SNPs were filtered using a minimum of 1 % MAF (average variant allele frequency < 0.99 and > 0.01). Finally, SNPs were filtered with a read coverage between 20 and 100 and a maximum number of missing data of 25 %. Individuals with more than 25 % missing data were not included in the analysis.

Population genetic analysis on variability and structure

The percentage of polymorphic loci (P %), inbreeding coefficient (F) expected (H_E), unbiased expected (uH_E) and observed (H_O) heterozygosity, were estimated using GENALEX v. 6.5 (Peakall & Smouse 2012).

Genetic differentiation (F_{ST}) between the 6 populations and test for Hardy-Weinberg equilibrium (HWE) within every population were performed with the GENEPOP package 3.4 (Raymond & Rousset 1995).

We also analysed the SNP dataset for the presence of isolation by distance (IBD) using the Mantel test implemented in GENALEX (Mantel 1967). Pairwise genetic distances among all individuals were plotted against all geographical distances among individuals. IBD is a common spatial genetic pattern in mobile and continuously distributed species (Wright 1943) and its existence may represent a challenge to the performance of clustering methods (Meirmans 2012).

The geographic distances connecting samples were represented by Euclidean (linear geographic) distances computed in QGIS (QGIS Development Team 2019).

The genetic structure of the populations of the sampled individuals ($n = 178$) was assessed based on a Bayesian clustering method, implemented in the software STRUCTURE v. 2.3, which clusters individuals into genetically distinct populations/groups (K) based on their allelic frequency at multiple loci (Pritchard et al. 2000). To illustrate the population genetic structure of hedgehogs, 10 independent runs of $K = 1- 5$ were carried out on the populations with 10^6 Markov chain Monte Carlo (MCMC) iterations and 10^5 burn-in period on the basis of independent allele frequencies and admixture ancestry model. The accurate number of

populations (K) was determined according to the ΔK formula (Pritchard et al. 2000) using the program STRUCTURE HARVESTER (Evanno et al. 2005). The islands of Lolland and Falster were combined into a single population due to a limited sample size and a clear genetic similarity between individuals from the two islands.

A multivariate ordination of individual genotypes was obtained by principal component analysis (PCA) using the software GENODIVE (Meirmans & Van Tienderen 2004) and plotted with the software PAST version 1.90 (Hammer et al. 2001).

Assessment of the demographic history

Tests for recent population declines or expansions in the population size were performed for every population with the program BOTTLENECK v. 1.2, after 15,000 iterations assuming an infinite allele model (IAM) according to a deficiency of the rare alleles and an excess of the heterozygosity (Luikart & Cornuet 1998; Luikart et al. 2010).

Estimation of potential anthropogenic effects on genetic variability of hedgehogs

To investigate whether the differences in genetic variability found between the hedgehog populations were associated with human population density in their area, road density and farmland density of the areas from which the studied hedgehogs derived, we fitted linear models in R (R Core Team 2019). The response variables were H_E , uH_E and F and the explanatory variables were human population density per km^2 , farmland per km^2 , and kilometres of roads per km^2 .

For each of the three response variables (H_E , uH_E and F) we first fitted models with a single explanatory variable at a time (i.e. three models for each of the three response variables). Then we fitted models that included all three explanatory variables as main effects, followed by a model that included road density and farmland density and the interactions between them. We tested the significance of the interaction term by comparing models that included the interaction term to those that did not include the interaction term, using an ANOVA test.

Results

Genotypes

Using the *Sau96I* restriction enzyme we were able to recover a total of 2.4 million high-quality SNPs. Following filtering for $\text{MAF} > 1\%$ estimated from the read coverage, a maximum of

25 % missing data, and a read coverage ranging from 20 to 100, resulted in 2902 applicable SNPs. Individuals containing less than 75 % of the selected SNPs were not included in the analysis and this resulted in 178 individuals used for studying the genetic variability.

The geographical sampling locations are shown in Fig.1, representing Jutland north of the Limfjord (JNL), Jutland south of the Limfjord (JSL), Funen (F), Zealand (Z), Lolland and Falster (LFA) and Bornholm (BH).

Fig. 1. Geographical overview of the samples.

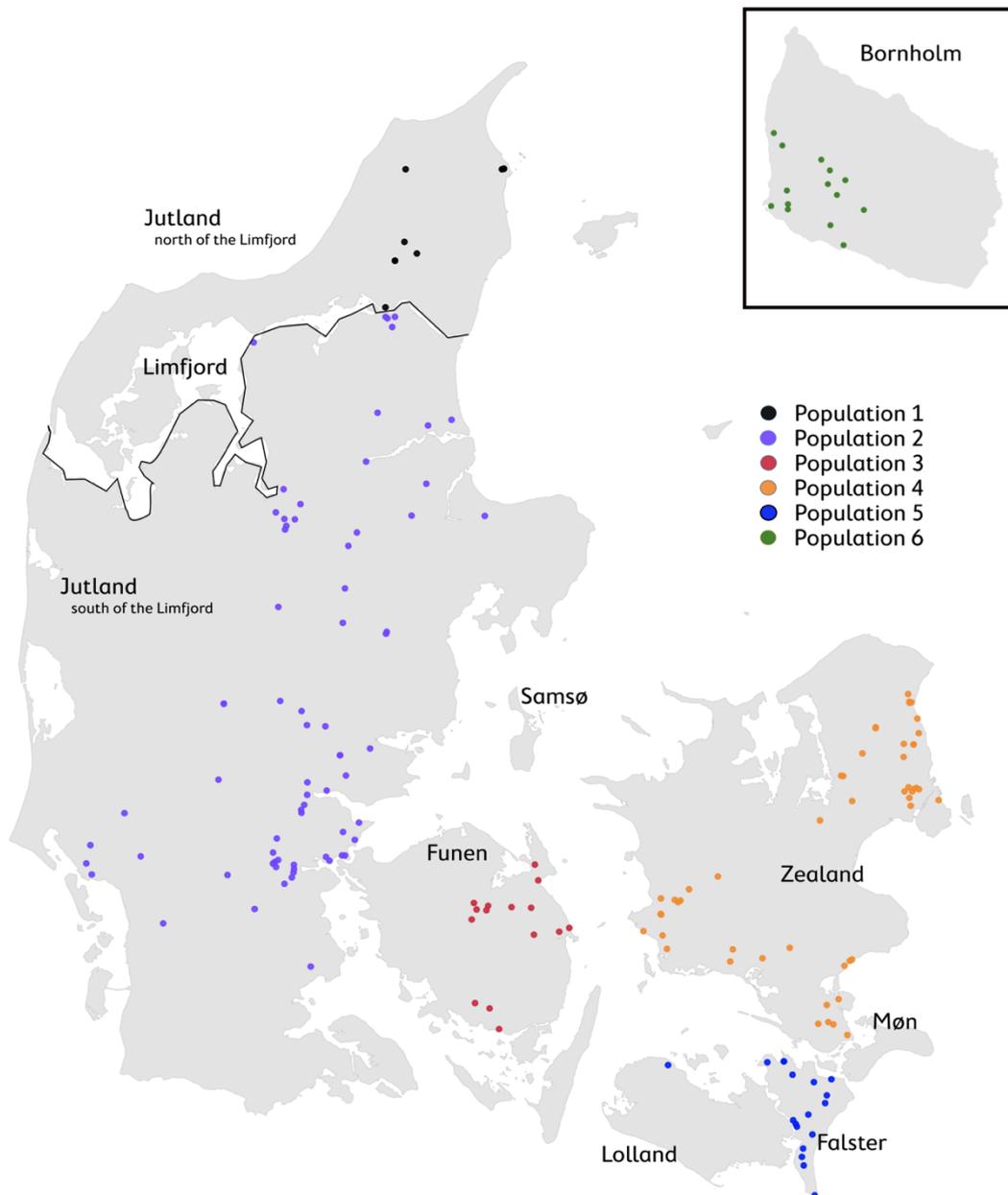


Fig.1. Map of Denmark indicating the locations of the 178 hedgehogs used in the study.

Genetic variability and structure

We found that the expected heterozygosity (H_E), unbiased expected heterozygosity (uH_E) and inbreeding coefficient (F), varied with location (Fig. 2). H_E showed marked variation among regions, with the lowest value reported on Bornholm and the highest in Jutland south of the Limfjord. The measure was very similar in Funen and Jutland north of the Limfjord. The same applied to the measures of uH_E . Of the three statistically significant F values (F , JSL, Z) the levels were similar between Jutland south of the Limfjord and Zealand, but markedly lower on Funen (Fig. 2, Table 2).

The genetic polymorphism increased according to the size of the regions, ranging from 54.44 % in BH to 94.79 % in JSL (Table 2).

Table 2. Table of genetic heterozygosity.

Population		Ho	He	uHe	F	% P	HWE TEST
Pop1	Mean	0.212	0.259	0.277	0.169	72.72%	NS
JNL	SE	0.004	0.004	0.004	0.008		
Pop2	Mean	0.293	0.316	0.318	0.097	94.79%	***
JSL	SE	0.004	0.003	0.003	0.004		
Pop3	Mean	0.253	0.252	0.261	0.017	79.08%	***
F	SE	0.004	0.004	0.004	0.006		
Pop4	Mean	0.206	0.212	0.215	0.096	87.08%	***
Z	SE	0.004	0.004	0.004	0.006		
Pop5	Mean	0.208	0.180	0.185	-0.075	67.73%	NS
LFA	SE	0.005	0.004	0.004	0.006		
Pop6	Mean	0.124	0.121	0.126	0.020	54.44%	NS
BH	SE	0.004	0.003	0.003	0.006		

Table 2. Table presenting the percent of polymorphic loci ($P\%$), inbreeding coefficient (F), expected (H_E), unbiased expected (uH_E) and observed (H_O) heterozygosity and test for Hardy-Weinberg equilibrium (HWE) for the six hedgehog populations studied.

Fig. 2. Plots of genetic heterozygosity and location.

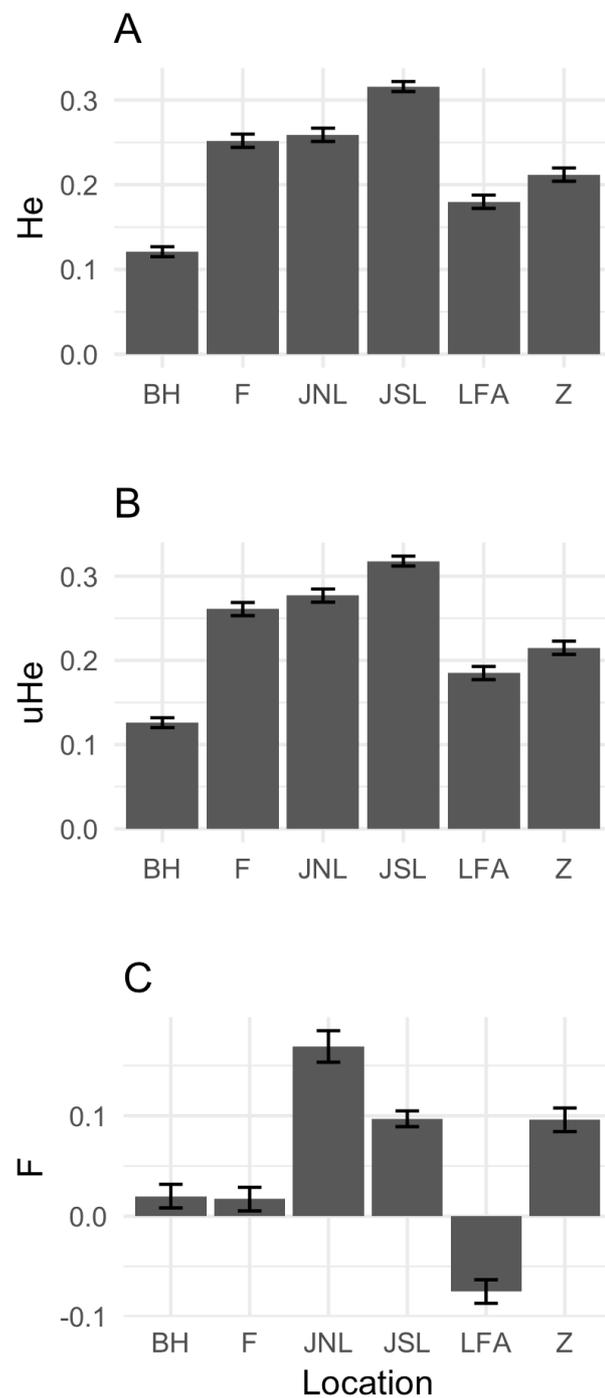


Fig. 2. Measures of (A) expected heterozygosity (H_E), (B) unbiased expected heterozygosity (uH_E) and (C) inbreeding coefficient (F), vary with location. Height of the bars indicate the estimated value and error bars represent the 95 % confidence intervals of these estimates. Locations are as follows: BH = Bornholm, F = Funen, JNL = Jutland north of the Limfjord, JSL = Jutland south of the Limfjord, LFA = Lolland and Falster and Z = Zealand.

The uH_E ranged from $uH_E = 0.126$ in BH to $uH_E = 0.318$ in JSL and H_E ranged from $H_E = 0.121$ in BH to $H_E = 0.316$ in JSL whereas, the H_O ranged from $H_O = 0.124$ in BH to $H_O = 0.293$ in JSL (Table 2).

We detected a significant deviation for HWE in JSL, F and Z and in all cases the deviations were due to a deficiency of heterozygotes which is also reflected by the positive F values which ranged from -0.0075 in LFA to 0.17 in JNL (Table 2).

We found that all the pairwise fixation index (F_{ST}) values between populations were highly significant ($p < 0.001$) with a F_{ST} range from 0.034 between JNL and JSL and $F_{ST} = 0.321$ between JNL and BH (Table 3), indicating that the six populations of hedgehogs tested, are genetically different.

Table 3. Pairwise F_{ST} values matrix.

Pop1	Pop2	Pop3	Pop4	Pop5	Pop6	
						Pop1
0.034						Pop2
0.146	0.108					Pop3
0.201	0.159	0.046				Pop4
0.237	0.191	0.063	0.030			Pop5
0.321	0.268	0.192	0.182	0.204		Pop6

Table 3. Pairwise F_{ST} values matrix, all the F_{ST} values were highly significant ($p < 0.001$).

The Bayesian clustering of the genotyped data assigned the highest posterior probability: Estimated Ln Prob of Data = -144614.8, Mean value of ln likelihood = -143612.8; Variance of ln likelihood = 1958.7 for $K = 3$. We found that the three clusters included: 1) Jutland north of the Limfjord (JNL) and Jutland south of the Limfjord (JSL); 2) Funen (F), Zealand (Z), Lolland and Falster (LFA); 3) Bornholm (BH).

The PCA was concordant with the clustering of STRUCTURE (Fig.3), the PC1 (Eigenvalues 19.54; variance explained; 79.24 %) and PC2 (Eigenvalues 5.12; variance explained; 20.76 %) and the convex hulls kept the three clusters well separated (Fig. 4).

The Mantel test for association between genetic and geographic distances within the populations was not significant for any of the six populations investigated (range $b = 0.006$ -0.08, $R^2 = 0.029$ -0.046, $0.062 < p < 0.1$).

Fig. 3. STRUCTURE analysis plot.

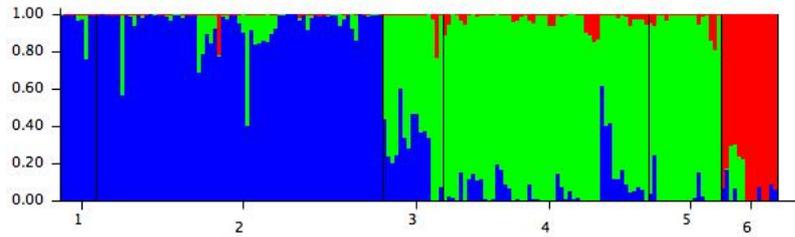


Fig.3. Plot of the STRUCTURE analysis.

Fig. 4. Principal component analysis.

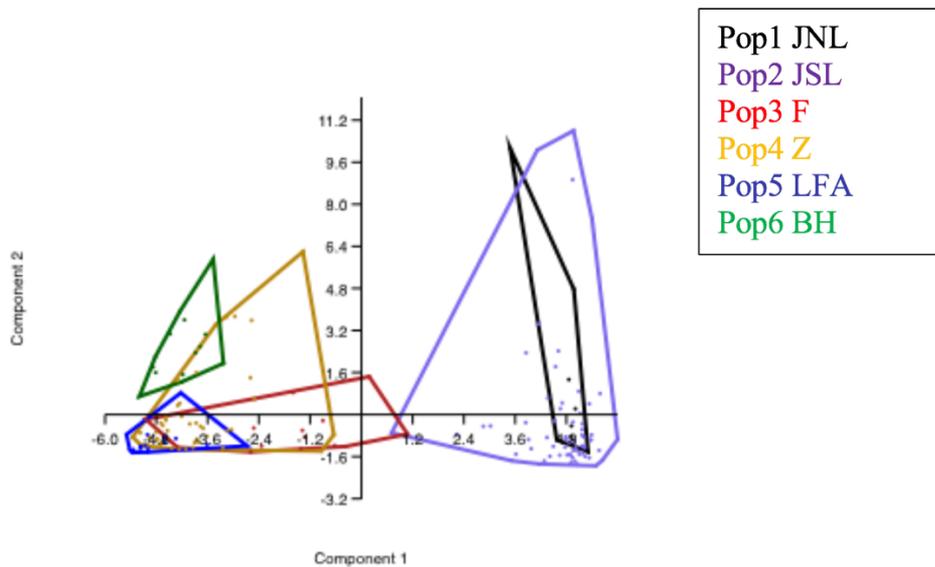


Fig. 4. Principal component analysis of the PC1 (Eigenvalues 19.54; variance explained; 79.24 %) and PC2 (Eigenvalues 5.12; variance explained; 20.76 %) and the convex hulls. The following colours are indicating the discovered populations: Pop1 (Black) Jutland north of the Limfjord, Pop2 (Purple) Jutland south of the Limfjord, Pop3 (Red) Funen, Pop4 (Orange) Zealand, Pop5 (Blue) Lolland+Falster, Pop6 (Green) Bornholm.

Demographic changes

We found no signs of recent increase or decline in the population in the six populations tested by use of the infinite allele model (IAM).

Estimation of potential anthropogenic effects on genetic variability of hedgehogs

Testing for effects of farmland density, population density and road density in the particular areas from where the hedgehogs derived, on the genetic variability observed in the study, we found no statistically significant associations between H_E and farmland density ($F[1,4] = 0.404$, $p = 0.560$), nor with road density ($F[1,4] = 0.787$, $p = 0.425$) or population density ($F[1,4] = 0.011$, $p = 0.922$), fitted as single main effects. A model fitted with both road density and farmland density terms as main effects showed no significant associations (Road density: $F = 0.737$, $p = 0.454$; Farmland density: $F = 0.746$, $p = 0.451$). A model fitted with all three explanatory variables furthermore failed to detect associations between the explanatory variables and H_E (Road density: $F = 0.959$, $p = 0.431$; Farmland density: $F = 0.970$, $p = 0.428$; Population density: $F = 1.904$, $p = 0.302$). We also tested whether there was a significant interactive effect between road and farmland density by comparing models with and without the interaction, but again found no significant relationship (ANOVA, $F = 0.288$, $p = 0.645$).

There were no statistically significant associations between uH_E and farmland density ($F[1,4] = 0.405$, $p = 0.559$), nor with road density ($F[1,4] = 0.935$, $p = 0.388$) or population density ($F[1,4] = 0.003$, $p = 0.958$), fitted as single main effects. A model fitted with both road density and farmland density terms showed no significant associations (Road density: $F = 0.89$, $p = 0.415$; Farmland density: $F = 0.81$, $p = 0.434$). A further model including all three explanatory variables failed to indicate effects on uH_E (Road density: $F = 1.209$, $p = 0.386$; Farmland density: $F = 1.100$, $p = 0.404$; Population density: $F = 2.073$, $p = 0.287$). We furthermore tested whether there was a significant interactive effect between road and farmland density by comparing models with and without the interaction, but detected no significant relationship (ANOVA, $F = 0.3477$, $p = 0.6152$).

We found no statistically significant associations between F and road density ($t = 0.136$, $p = 0.898$, $df = 4$) or population density ($t = 0.597$, $p = 0.583$, $df = 4$) fitted as single main effects. However, we found a statistically significant association between F and farmland density ($t = 3.099$, $p = 0.0363$, $df = 4$). F decreases with increased farmland density (with a factor 1.0315). A model fitted with farmland density and road density terms as main effects also showed a significant association between farmland density and F (Road density: $t = 0.993$, $p = 0.3940$; Farmland density: $t = 3.239$, $p = 0.0479$, $df = 3$). When we fitted a model with all three explanatory variables, we only found a weak effect on farmland density (Road density: $t = 2.432$, $p = 0.1355$; Farmland density: $t = 3.952$, $p = 0.0585$; Population density: $t = 1.991$, $p = 0.1848$, $df = 2$). Lastly, we tested whether there was a significant interactive effect between

road and farmland density by comparing models with and without the interaction, but again found no significant relationship (ANOVA, $F = 0.0304$, $p = 0.8776$).

Discussion

Genetic variability and structure

Previous genetic studies on hedgehogs in Europe based on microsatellite techniques, have found a mean H_E ranging between 0.239 and 0.6872. A calculated mean H_E of 0.239 and a mean H_O of 0.218 ($n = 42$) was reported in an isolated population of hedgehogs in Regent's Park, London (O'Reilly 2016), while a mean H_O of 0.7 ($n = 160$) was determined in a study from Oxfordshire, UK (Becher & Griffiths 1998). A mean H_E of 0.6872, a mean H_O of 0.695 and a mean F of 0.0686 was determined in hedgehogs from the Czech and Slovak Republics ($n = 131$) (Bolfikova & Hulva 2012). Mean H_E ranged between 0.569-0.627 ($n = 147$) and the mean F ranged between -0.006-0.070 for three genetic clusters in Zurich, Switzerland (Braaker 2017). In our research based on the GBS technique, we found a mean H_E varying between 0.121-0.316 and a mean H_O varying between 0.124-0.293 for the Danish hedgehogs studied (Table 2). As Denmark is a relatively small area compared to e.g. continental Europe, with an isolated island structure, and thereby has a reduced gene flow, a lower heterozygosity would be expected for the Danish hedgehogs. The peninsula of Jutland south of the Limfjord is connected to Germany, which could potentially have caused a higher gene flow, but we still found inbreeding and low heterozygosity in this population ($H_O = 0.293$, $H_E = 0.316$, $F = 0.097$). However, individuals from Jutland south of the Limfjord did in fact have the highest measures of heterozygosity (H_E , H_O and uH_E) of all the areas tested in the present study (Fig. 2, Table 2). Furthermore, in comparison to using SNPs with only two possible alleles, the microsatellite approach in the previous studies may also overestimate heterozygosity due to the polymorphic nature of the microsatellites (Putman & Carbone 2014).

Both the STRUCTURE analysis (Fig. 3) and PCA plot (Fig. 4) in the present study were concordant, and facilitated the subdivision of the Danish hedgehog population into three well-defined clusters: Jutland north of the Limfjord (JNL) and Jutland south of the Limfjord (JSL); Funen (F), Zealand (Z), Lolland and Falster (LFA); Bornholm (BH). It is quite evident that the population in Jutland (JNL, JSL) forms one single cluster whereas all the remaining islands tested, apart from BH, form the second cluster. Lastly, the STRUCTURE analysis plot (Fig. 3) is clearly indicating an admixed structure on the island of F. The plot (Fig. 3) is also supported by the PCA plot (Fig. 4), which shows that JSL and JNL are overlapping and are well separated

from F, Z and LFA which are forming a second cluster. Lastly, the island of BH is separated from the second cluster (F, Z, LFA) even if partially overlapping with it, and is clearly separated from the first cluster (JSL, JNL). The island of Bornholm, which was previously connected to the continent around northern Germany, became an island in late Preboreal approximately 8000 BC (Aaris-Sørensen 1998). The rest of Denmark was part of a large continent connected with current areas such as UK and southern Sweden (Aaris-Sørensen 1998), but was transformed into islands and the peninsula of Jutland around 6000-6500 BC (Aaris-Sørensen 2009), when the North American ice shield melted and made the oceans rise. At that point in time, Denmark was surrounded by the Littorina Sea, Lolland and Falster was still connected, and the area south and north of the Limfjord and Djursland as well as northern Zealand consisted of archipelagos (Aaris-Sørensen 1998). It was not until around 6000 BC that the current geography of Denmark was shaped. Previous research indicates that Lolland and Falster was divided by the sound Guldborgsund around 4000 BC (Bennike & Jensen 2011), but may have been periodically connected up until 1000 AD (Jakobsen 1987), which could explain the non-significant difference of F_{ST} found between the two islands before we decided to merge them into one population. Jutland north and south of the Limfjord have regularly been connected by different isthmuses closing off the western entrance to the Limfjord from the North Sea from around 1200 AD. In 1863 it was decided to artificially maintain an opening between the Limfjord and the North Sea (Bennike et al. 2019). The periodical connection between Jutland north and south of the Limfjord could have influenced the genetic cluster found between those two populations of hedgehogs.

The F_{ST} values were all highly significant, however, due to the fact that several of the populations investigated are not in HWE the F_{ST} values should be interpreted with caution, as one of the assumptions for a correct estimate test is that the populations which are compared, are panmictic. However, Table 3 is clearly showing that the F_{ST} values are higher between the islands.

Population 1 (JNL), 5 (LFA) and 6 (BH) had a low genetic variability (Table 2), but the HWE test was non-significant, which indicates that we were unable to detect inbreeding or further genetic substructuring due to habitat fragmentation for these populations. However, these three populations did have considerably smaller sample sizes than population 2 and 4, which could have affected the results. As an example, population 1 had an inbreeding coefficient (F) of 16.9 %, but the HWE test was still negative. This could be due to the small sample size or the large standard errors of H_E and H_O .

Population 2 (JSL), 3 (F) and 4 (Z) also showed a low genetic variability (Table 2), and the significant HWE tests indicate that the populations from these areas are inbred and/or fragmented into subpopulations under the influence of the Wahlund effect (Garnier-Géré & Chikhi 2013).

Despite the deviation from HWE found in several of the populations investigated (Table 2), the STRUCTURE analysis failed to find further substructuring, and the Mantel test failed to find an isolation by distance effect. This lack of correlation between the genetic distance and the geographic distance between individuals could be due to the presence of barriers to geneflow such as habitat fragmentation, which could have confounded the expected isolation by distance pattern. The significant deviation from HWE observed in three of the populations investigated could reinforce the hypothesis, as the deviation from HWE could be due to further substructuring of the populations investigated (Wahlund effect) which produces a heterozygosity deficiency due to the lack of panmixia, as seen in hedgehog populations, where competition for the favor of females often occur. Additionally, female hedgehogs are selective of their mates, which often results in courtship without mating (Morris 2018).

A likely explanation for the lack of isolation by distance (IBD) patterns found, could be the effect of humans relocating hedgehogs into foreign habitats. During the past 20-30 years, the rehabilitation of sick, orphaned and injured wild hedgehogs has become an established practice in many western European countries. Denmark has a number of working hedgehog rehabilitation centres, where volunteers care for the hedgehogs with the purpose of releasing the surviving individuals back into the wild. In 2013 the Danish Nature Agency prepared guidelines for the care of wildlife, instructing rehabilitators to refrain from moving mammals over water, and ensuring that rehabilitated wildlife would be released back into their original habitat (Danish Nature Agency 2013). Injured wildlife from e.g. Jutland north of the Limfjord, should therefore only be admitted to a wildlife rehabilitation centre north of the Limfjord and be released into the original habitat. Only recently, in 2019, have the Danish authorities established legal frameworks and monitoring programs for the practice of wildlife rehabilitation. The practice before 2013 was to transport wildlife in need of care to the nearest rehabilitation centre, which could be situated far away from the original habitat. Often the animals would be released near the rehabilitation centre. There are previous examples of hedgehogs from e.g. the small island of Ærø being cared for in the northern part of Zealand 200 km away, crossing the seas of the South Funen Archipelago and the Great Belt, most likely because people had brought the sick hedgehog with them when returning home vacation (pers. comm. Danish Animal Welfare Society). Furthermore, Kristiansson (1981) showed that

intentional introductions of hedgehogs could have influenced the distribution of hedgehogs in Sweden, Norway and Finland. The anthropogenic effects on hedgehog genetics in Denmark could largely be explained by the transportation of hedgehogs between different parts of Denmark.

Effective population size and population bottleneck and expansion

The low level of genetic variability and the significant and positive F values found for some of the populations (JSL, F, Z) can be explained by inbreeding, genetic substructure and extremely low N_e or a combination between these factors which could be caused by habitat fragmentation and/or the large amount of farmland in Denmark. As intensified agricultural practises increase, arable land is gradually becoming a less suitable habitat for hedgehogs. The decline in the hedgehog population of the UK has even been found to be more severe in rural areas than urban (SoBH 2018). Two thirds of the area of Denmark is arable land (Statistics Denmark 2017). In comparison, the share of total area by type and land cover in percentage of the EU countries show, that the amount of cropland is particularly high in Denmark (50.2 %) compared to e.g. Austria (15.3 %), United Kingdom (19.7 %), Slovakia (26.6 %) and the Czech Republic (32 %) (Eurostat 2015), where previous studies on hedgehog genetics found remarkably higher genetic variability using microsatellites (Becher & Griffiths 1998; Bolfikova & Hulva 2012; Braaker 2017). We found an effect of farmland density on the inbreeding coefficients (F) in our study, but the effect was surprisingly that F decreased with increasing farmland density. This may be due to the tendency for a lower degree of road-associated fragmentation in farmland areas and less traffic in general. Or perhaps because the hedgehogs, which are able to survive in this habitat type, can move more freely in their search for mates, because the limitations of movement primarily present in urban areas such as buildings, fences and traffic-laden roads, are less pronounced in farmland areas. Further research is needed to understand this effect.

Habitat fragmentation can cause founder effects (Provine 2004) and, because hedgehogs have relatively small home ranges and are not dispersing far from their birthplace, they are vulnerable to habitat fragmentation and barriers of movement in general. Consequently, a conservation campaign has been established in the UK, where citizens are encouraged to make holes in their fences to increase garden connectivity for the hedgehogs. Roads as barriers causing habitat fragmentation are also a challenge for hedgehogs, as they are often killed in traffic when crossing roads, especially during the mating season, where males increase their home ranges in search for mates (Morris 2018). As a supplementary measure to the Mantel

test, we tested the possible effects of road density, as a measure of habitat fragmentation in the area from which the hedgehogs derived, on the genetic variability found. We failed to find an association between road density and F , H_E and uH_E .

We also tested whether the human population density per area for each population of hedgehogs studied, had an effect on F , H_E and uH_E . Population 2, 3 and 4, where we found inbreeding and/or subpopulations, had the highest number of citizens per area. This measure may indicate that hedgehogs in these areas are under stronger influence of anthropogenic effects caused by factors such as more cars and traffic and more construction sites replacing hedgehog habitats. However, we failed to find an association between population density and genetic variability in the hedgehogs studied.

The tests for effects of population density, farmland density and road density were based on a small sample size ($n = 6$ populations), which has likely influenced the statistical power of the tests.

The software BOTTLENECK 1.2. failed to detect signs of population bottlenecks or increase in population size. However, the software only detects decreases or increases in population size, which has occurred recently (within $0.2 N_e$ to $0.4 N_e$ generations). Therefore, we cannot reject the possibility that the population is declining and or have declined drastically before the scope of 0.2 to $0.4 N_e$ generations.

Conclusions

Adapting the GBS technique with the application of 2902 SNPs per individual to investigate the genetics of Danish hedgehogs, we found a low genetic variability ($H_O = 0.124-0.293$). This indicates that the population is inbred and/or highly fragmented. The population of hedgehogs in Denmark must therefore be negatively influenced by one or more factors, such as landscape fragmentation, roads, intense agricultural practises or other anthropogenic effects. However we failed to find support for these drivers in our analysis. Nevertheless, given the lack of knowledge on the population status of Danish hedgehogs, we believe that future research on hedgehog genetics should focus on the effects of low individual genetic heterozygosity to determine the impact of inbreeding on individual fitness including indicators such as dental health, parasitic load, toxicology and prevalence of cancer.

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Manuscript III

European hedgehogs (*Erinaceus europaeus*) as a natural reservoir of methicillin-resistant *Staphylococcus aureus* carrying *mecC* in Denmark



European hedgehogs (*Erinaceus europaeus*) as a natural reservoir of methicillin-resistant *Staphylococcus aureus* carrying *mecC* in Denmark

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Abstract

The objective of the study was to:

- 1) Determine the dissemination of methicillin-resistant *Staphylococcus aureus* (MRSA) in European hedgehogs on large-scale nationwide study in Denmark.
- 2) Investigate determinants of MRSA carriage in hedgehogs.
- 3) Determine the potential for zoonotic transmission of *mecC*-MRSA from hedgehogs to humans.
- 4) Characterize the detected *mecC*-MRSA on both a phenotypic and molecular level.

Nasal swabs were taken from 188 dead hedgehogs collected by volunteers throughout Denmark to determine the prevalence of MRSA. Additionally, 16 hedgehog rehabilitators were tested for potential zoonotic transmission of MRSA from hedgehogs to humans.

The swabs were incubated in tryptic soy broth supplemented with 6.5 % NaCl, followed by spread of 10 µl on Brilliance MRSA 2 agar. One presumptive MRSA colony from each plate was subcultured on 5 % blood agar. All *S. aureus* subcultures were verified by a PCR assay detecting *mecA*, *mecC*, *lukF-PV*, *scn*, and *spa*, followed by *spa* typing.

A total of 114 (61 %) hedgehogs carried *mecC*-MRSA. The detected *mecC*-MRSA belonged to two genetic lineages CC130 (*spa*-types: t528, t843, t1048, t3256, t3570, t6220, t17133) and CC1943 (*spa*-types: t978, t2345, t3391, t8835, t16868), of which CC130: *spa*-type t843 was the most prevalent *spa*-type, accounting for 52 %. The prevalence of *mecC*-MRSA in the hedgehogs was similar regardless of cause of death, sex, region and habitat type. None of the hedgehog rehabilitators carried MRSA.

This nationwide study confirms a high prevalence of *mecC*-MRSA in hedgehogs, which could serve as a natural reservoir for this specific type of MRSA. Furthermore, our study found no sign of zoonotic transmission of *mecC*-MRSA to hedgehog rehabilitators.

Introduction

Staphylococcus aureus is a commensal bacterium with the potential to cause severe infections in humans. Methicillin-resistant *S. aureus* (MRSA) are resistant to most β -lactam antibiotics, due to the expression of additional penicillin binding proteins (PBPs) with low affinity to β -lactams, which are encoded by either *mecA*, *mecB* or *mecC* genes, of which *mecA* and *mecC* are carried in mobile genetic elements, referred to as staphylococcal cassette chromosome *mec* (SCC*mec*) (Paterson et al. 2014a). MRSA is a major cause of hospital-associated infections in Europe (Kock et al. 2010), and since the 1990s there has been a rise in community-associated MRSA infections among people with no apparent risk factors for contracting MRSA (Aires-de-Sousa 2017). During the last decade, livestock-associated MRSA has additionally been recognized as a third major cause of MRSA infections in humans (Graveland et al. 2011; Kock et al. 2010; Petersen et al. 2013a).

The *mecA* gene predominates in human MRSA isolates, and it was not until 2011 that the *mecC* gene was described in *S. aureus* from humans and dairy cattle from Denmark, England, Ireland and Scotland (Garcia-Alvarez et al. 2011; Shore et al. 2011). Subsequently, *mecC*-MRSA was detected in a wide range of domesticated animals of Europe, such as swine, small ruminants and horses in Denmark (Angen et al. 2017b; Eriksson et al. 2013; Islam et al. 2017), and cattle in the UK (Paterson et al. 2014b). Additionally, zoonotic transmission of *mecC*-MRSA from livestock to humans has been detected on several occasions (Goerge et al. 2017; Harrison et al. 2013; Paterson et al. 2013; Petersen et al. 2013a). The first assumption was therefore that *mecC*-MRSA had a livestock reservoir, but the detection of *mecC*-MRSA in several species of European wildlife (Aires-de-Sousa 2017; Bengtsson et al. 2017; Carson et al. 2012; Gomez et al. 2014; Gomez et al. 2015; Himsworth et al. 2014; Loncaric et al. 2014; Monecke et al. 2016; Monecke et al. 2013; Mrochen et al. 2017; Porrero et al. 2013; Porrero et al. 2014) and in urban waste water (Porrero et al. 2014) and river water (Concepcion Porrero et al. 2014) indicates otherwise. The highest prevalence of human *mecC*-MRSA cases have been found in Denmark, accounting for 1-2 % (30-50 cases annually) of all human MRSA cases, where they primarily cause skin and soft tissue infections (Petersen et al. 2013b). In contrast to most MRSA isolates of human origin, *mecC*-MRSA does not seem to carry the ϕ Sa3 phage-encoded modulators of the human innate immune responses, including SAK (*sak*), CHIPS (*chp*), and SCIN (*scn*), which furthermore indicates a non-human origin (Harrison et al. 2013).

MRSA in European hedgehogs

The western European hedgehog (*Erinaceus europaeus*, hereafter referred to as “hedgehog”) is a small, spiny mammal which can be found throughout western Europe and New Zealand (Reeve 1994). Hedgehogs are increasingly inhabiting areas with human activity, for instance gardens in residential areas and rural villages (Hubert et al. 2011; van de Poel et al. 2015). Garden owners are generously supplying food and water for hedgehogs (Morris 2014). The feeding of hedgehogs, combined with the tendency for hedgehogs to become habituated to human presence, makes the hedgehog one of the few wild mammals people are prone to come into physical contact with.

The first description of penicillin-resistant *Staphylococcus aureus* in hedgehogs was made in 1964 (Smith & Marples 1964). Smith & Marples (1964) isolated *S. aureus* in 40 % of the nasal swabs, 63 % of skin surface swabs and 71 % of the paw swabs, of 35 hedgehogs in New Zealand. A large proportion (79-92 %) of these samples were resistant to penicillin. Smith (1965) furthermore reported an *S. aureus* prevalence of 85 % in 59 hedgehogs from New Zealand and showed that 86.3 % of the strains were resistant to penicillin. Recently, Bengtsson et al. (2017) detected *mecC*-MRSA in 64 % of 55 hedgehogs from five counties in Sweden, of which most had died in care at wildlife rehabilitation centres. This prevalence of *mecC*-MRSA in hedgehogs seems extraordinarily high and indicates either dissemination of *mecC*-MRSA at the wildlife rehabilitation centres or hedgehogs being an important natural reservoir for *mecC*-MRSA, which was also suggested by Bengtsson et al. (2017).

The aim of the research

Previous studies on MRSA in wildlife have been limited by factors including small sample sizes, small geographical ranges, or by the use of samples from weak animals that have been in close contact with other wildlife species and humans during care at wildlife rehabilitation centres. Here we ameliorate these limitations by carrying out a large-scale nationwide sampling of wild hedgehogs in Denmark, aiming to discover whether the high prevalence found by Bengtsson et al. (2017) in Sweden would be confirmed in a large-scale nationwide study.

Materials and Methods

We established a nationwide citizen science project in Denmark to collect dead hedgehogs for a project aiming to understand more about hedgehog ecology. Volunteers were recruited via local and national media and a project website. Denmark consists of the large peninsula Jutland

and several islands of differing sizes. The larger islands are connected by 0.75-17 km long bridges, which hedgehogs are unlikely to cross, isolating the local hedgehog populations. We strived to obtain samples from all of these regions. As a result of these efforts, citizens collected 697 hedgehogs throughout Denmark between May and December 2016. These animals were either found dead in traffic, from natural causes in the wild, or had died in care at wildlife rehabilitation centres.

In addition to collecting the dead animal, the volunteers were instructed to record the date and location of the find and deliver the hedgehog to the nearest of 26 collection stations, distributed nationally. All hedgehog carcasses were individually collected and sealed. Volunteers housing the collection stations emptied the collection bins daily, and they stored the hedgehog carcasses in local freezers at -20°C before transportation to university laboratories, where they were thawed and necropsied. The necropsies took place from August 2016 to May 2018 and formed the basis for the present study.

Sampling for detection of methicillin-resistant *S. aureus* in hedgehogs

A representative subsample of the collected hedgehogs (n = 188) was selected based on their geographical location and tested for the presence of MRSA. To obtain a suitable sample for MRSA testing, a nasal swab was obtained from each individual just after thawing. A polyester tipped sterile applicator with a tip diameter of 1.98 mm (Puritan 25-1000 1PD) was used to swab the nares of the dead hedgehogs. The used applicator tip was subsequently placed in a sterilised 5.0 ml Eppendorf tube containing 700 µl of PBS and 300 µl of glycerol 50 %, and was stored at -80°C.

Sampling for detection of MRSA in hedgehog rehabilitators

To examine whether MRSA was present in humans with close contact to hedgehogs, 16 study participants working at wildlife rehabilitation centres under the Danish Animal Welfare Society were sampled from nose and tonsils in November 2017, and tested as described before by Angen et al. (2017a). A short questionnaire about demographics was used to collect data from each participant (e.g., demographic characteristics, contact to wildlife, food animals, pets and medical history). Data collection was approved by the Danish Data Protection Agency (protocol no. 2001-14-0021).

Isolation and characterization of MRSA

The samples obtained from hedgehogs and wildlife rehabilitators were analysed for presence and characterization of MRSA at the National Reference Laboratory for Antimicrobial Resistance at Statens Serum Institut as described previously (Islam et al. 2017; Stegger et al. 2012). Simpson's diversity index (Simpson 1949) was used to quantify *spa*-type diversity.

Antimicrobial susceptibility testing was performed by minimum inhibitory concentration (MIC) determination using a custom-made panel (DKSSP2, TREK Diagnostics), including 17 antimicrobials (penicillin, ceftazidime, ceftazidime/avibactam, ceftazidime/meropenem, ceftazidime/meropenem/avibactam, ceftazidime/meropenem/avibactam/ceftazidime, erythromycin, clindamycin, tetracycline, rifampicin, gentamicin, kanamycin, fusidic acid, sulfamethoxazole/trimethoprim, linezolid, mupirocin, vancomycin, daptomycin, norfloxacin). Interpretation of antimicrobial resistance was based on The European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints. For kanamycin and norfloxacin the breakpoints of Clinical and Laboratory Standards Institute (CLSI) were used. *S. aureus* ATCC 29213 was included as quality control of MIC determination.

Habitat classification

To assign the main habitat type to each hedgehog, all habitat types were extracted within a 500 m radius around where a hedgehog was found. This area is roughly equivalent to a large hedgehog home range (Reeve 1994). The habitat classes were extracted using CORINE land cover data with a 100 x 100 m resolution (CLC 2012, Version 18.5.1). CORINE land cover data describes habitat types derived from satellite imagery divided into artificial surfaces: industry, agricultural areas, forest and semi-natural areas, wetlands and water bodies. For each area around which a hedgehog was found, habitat types were extracted in R using the raster package (Hijmans 2018). Afterwards, the habitat types were reclassified as "urban", "rural" or "other" (See Supporting information Table 1 for further information). For further calculations, focus was on the percentages of urban versus rural, excluding other classes. The categorization of "urban" or "rural" was based on the highest percentagewise representation of the two categories for each individual hedgehog. Four individuals were excluded from the following analyses because their percentagewise representations were equally distributed between "rural" and "urban".

Determinants of MRSA carriage

To investigate whether the detection of MRSA in a hedgehog was associated with sex, the region or habitat type in which it was found, or cause of death, we fitted generalized linear

models (GLMs) in R (R Development Core Team 2018) with binomial errors and a logit link function. The binary response variable was the detection or not of MRSA and the explanatory variables were sex (female/male), region (a categorical variable), habitat (urban/rural) and cause of death (a 2-level categorical variable: in-care and natural/roadkill). We had small sample sizes from some regions and therefore we collapsed our region variables into four broader regions: 1) Jutland (north and south combined), 2) Lolland and Zealand together, 3) Funen, and 4) the islands of Bornholm, Møn, Samsø and Falster. We first fitted a maximal model including all explanatory variables and all two-way interactions between them (sex, cause of death, habitat, region). We then sequentially removed non-significant terms, interaction terms first and starting with the least significant, until we obtained a minimal adequate model where all remaining terms were significant (Crawley 2013). We tested significance of term deletions with Chi-squared tests using the *dropterm* function from the MASS package (Venables & Ripley 2002).

Animal Research Ethics

Ethical approval was not required for this research because the hedgehogs used in the study had already died of natural causes either in the wild or in care at a hedgehog rehabilitation centre.

Results

Of the 697 hedgehogs collected throughout Denmark, we selected a subsample of 188 representing the different regions of Denmark (Table 1 and Fig. 1). The sex distribution of the subsample tested for MRSA was 102 males, 56 females, and 30 individuals of unknown sex. Causes of death were 98 road-kills, 25 dying from natural causes in the wild, 16 dying in the wild from unknown causes and 49 dying in care. We found that 114 (61 %) individuals carried *mecC*-MRSA and none carried *mecA*-MRSA. None of the isolates carried the genes encoding Panton-Valentine leukocidin (*lukF-PV*) or the modulator of the human innate immune response SCIN (*scn*).

Fig 1. Map of MRSA distribution.

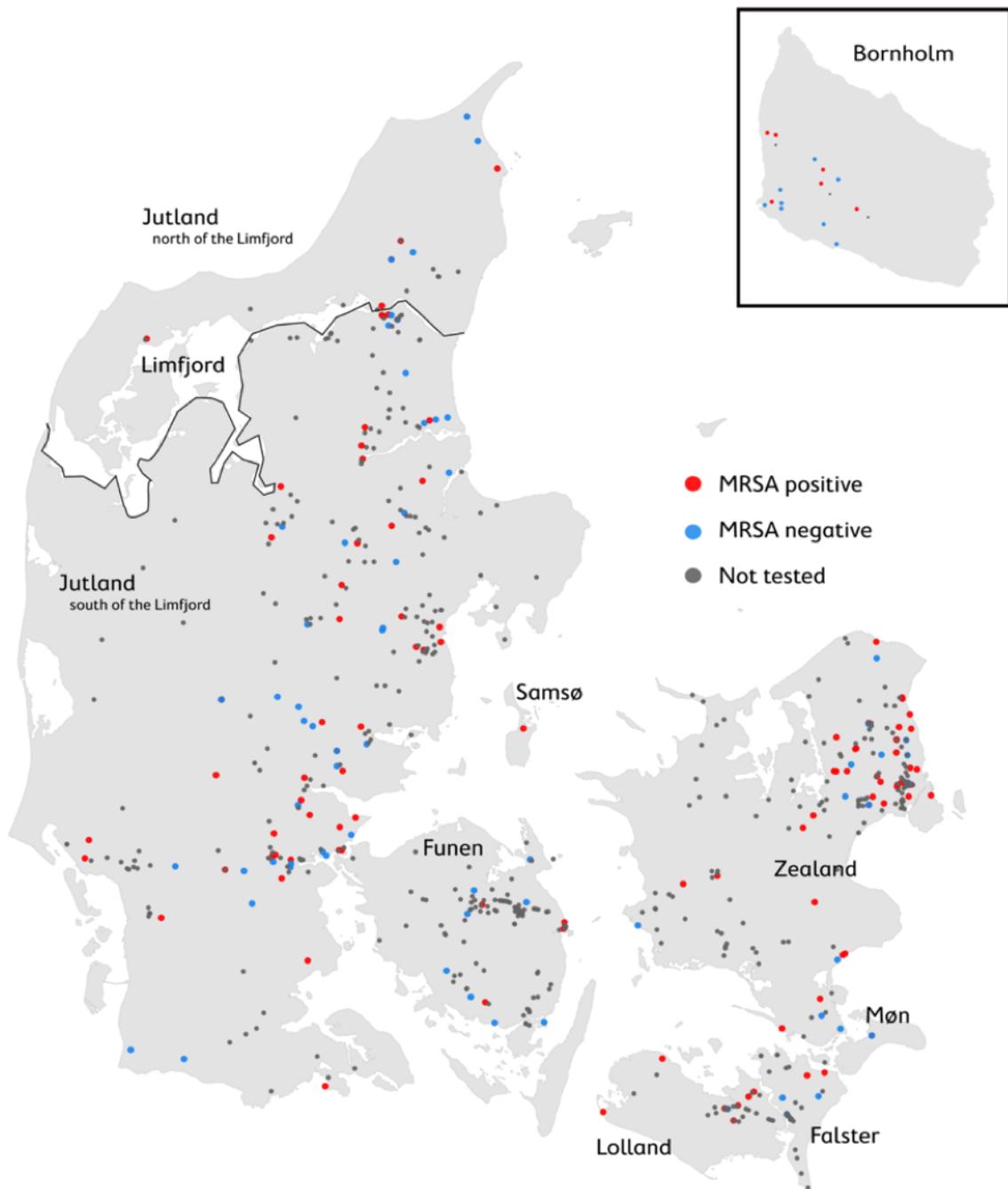


Fig 1. The distribution of the 697 collected, dead hedgehogs across Denmark. Each point represents an individual collected hedgehog. Grey points indicate that the individual was not MRSA tested, blue points indicate individuals that tested negative for MRSA, while red points indicate those that tested positive for *mecC*-MRSA.

mecC-MRSA was present in animals from all areas and islands investigated (Table 1).

Table 1. Occurrence of *mecC*-MRSA, CC-types and *spa*-types.

Region	No. tested/no. collected (%tested)	Prevalence of <i>mecC</i> -MRSA			<i>spa</i> -types			D
		CC130	CC1943	Total	CC130	CC1943		
Zealand	56/187 (30%)	46%	29%	75%	t843	t978, t3391, t8835	0.54	
Funen	14/121 (12%)	29%	14%	43%	t528, t843	t3391	0.73	
Jutland south of the Limfjord	79/277 (28%)	37%	19%	56%	t528, t843, t3256, t3570, t6220, t17133	t978, t2345, t3391	0.70	
Jutland north of the Limfjord	8/20 (40%)	13%	38%	50%	t843	t978, t2345, t8835	1.00	
Bornholm	14/18 (78%)	14%	29%	43%	t843	t8835	0.53	
Møn	2/2 (100%)	50%	0%	50%	t843		NA	
Lolland	10/36 (28%)	60%	20%	80%	t843, t1048	t978, t3391	0.64	
Falster	4/30 (13%)	25%	25%	50%	t1048	t978	1.00	
Samsø	1/1 (100%)	0%	100%	100%		t16868	NA	
Total	188/697 (27%)	37%	23%	61%	t528, t843, t1048, t3256, t3570, t6220, t17133	t2345, t3391, t8835, t16868, t978	0.69	

Table 1. An overview of the results found when testing 188 dead hedgehogs for MRSA. The results are divided into areas of Denmark that are isolated from one another by the sea. Abbreviations: MRSA, methicillin-resistant *Staphylococcus aureus*; CC, clonal complex; D, Simpson's index of diversity; NA, not applicable.

Characterization of the detected MRSA

The detected *mecC*-MRSA belonged to two genetic lineages CC130 (n = 70) and CC1943 (n = 44) and 12 different *spa*-types. The *spa*-types t528, t843, t1048, t3256, t3570, t6220, t17133 were associated with CC130, and the *spa*-types t978, t2345, t3391, t8835, t16868 were associated with CC1943. The prevalence of these CC- and *spa*-types appeared to vary geographically (Fig. 2). CC1943 and CC130 were found in all areas, except for Møn (n = 2), which only had CC130. The most frequent *spa*-types were t843 (n = 59 out of 114, 52 %) followed by t978 (n = 22 out of 114, 19 %). Two new *spa*-types were described (t16868 on Samsø and t17133 in Jutland south of the Limfjord). The largest number of different *spa*-types was found in Jutland south of the Limfjord (n = 9), which also contributed with the largest sample size (n = 79). The *spa*-types t3256, t3570, t6220, t17133 were only found in this area of Denmark.

Fig 2. Map of CC-type and *spa*-type distribution.

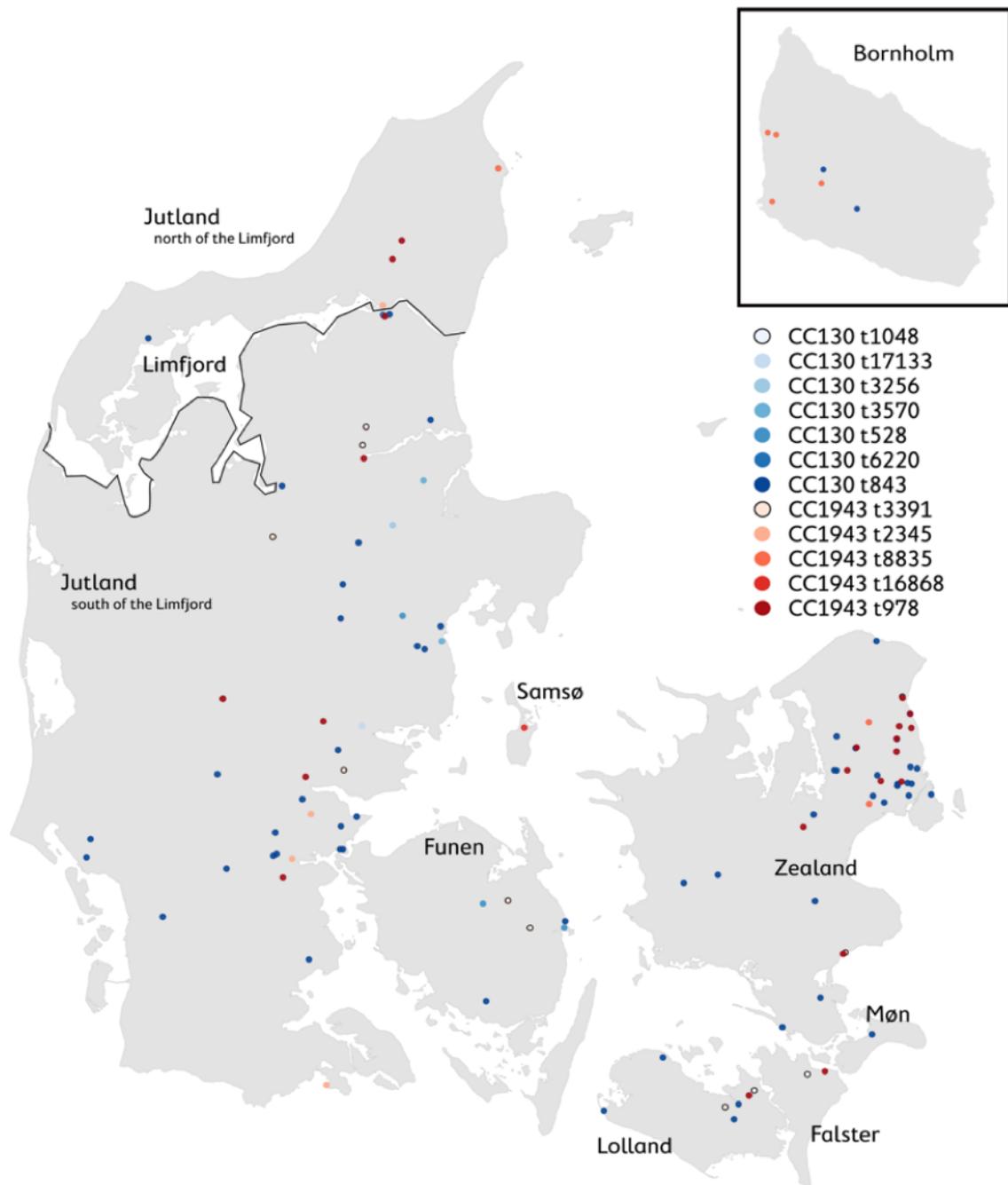


Fig 2. The distribution of the observed CC-types and *spa*-types of the 114 individuals carrying *mecC*-MRSA in Denmark.

When comparing the distribution of CC-types between the two largest coherent areas of Denmark, Jutland south of the Limfjord ($n = 79$) and Zealand ($n = 56$) representing western and eastern Denmark, respectively, the distribution of CC130 and CC1943 was similar (66 % and 34 % in Jutland south of the Limfjord vs. 62 % and 38 % on Zealand). However, the *spa*-

type diversity was higher in Jutland south of the Limfjord (Simpson's $D = 0.70$) than on Zealand (0.54) (Table 1). The two areas shared three *spa*-types (t843, t978 and t3391).

A larger proportion of males ($n = 67/102$, 66 %) than females ($n = 31/56$, 55 %) were *mecC*-MRSA positive, however, the difference was not found to be statistically significant. Of the individuals with unknown sex, 16 out of 30, 53 % tested positive for *mecC*-MRSA.

Neither were any statistically significant differences observed in the distribution and prevalence of MRSA based on cause of death: Individuals dying in care ($n = 28/49$, 57 %), individuals dying in the wild from natural causes ($n = 15/25$, 60 %), road-killed individuals ($n = 60/98$, 61 %), individuals dying in the wild from unknown causes ($n = 11/16$, 69 %).

Habitat analysis

Our habitat analyses showed that 56 % ($n = 103$) of the tested individuals resided in rural habitats and 44 % ($n = 81$) in urban habitats, defined as industrial areas or residential areas, smaller or larger cities. Of the total 111 individuals with precise location data carrying *mecC*-MRSA, 52 % ($n = 58$) lived in rural habitats and 48% ($n = 53$) in urban habitats. Of the 73 individuals that tested negative for MRSA, 62 % ($n = 45$) lived in rural habitats and 38 % ($n = 28$) in urban habitats.

Modelling determinants of MRSA occurrence

The minimal adequate model retained none of the explanatory variables, and the prevalence of MRSA was thus adequately explained by a model including only the intercept (0.454 ± 0.174 , expressed on the scale of the linear predictor (logit) used in the GLM). On the natural scale this represents a prevalence of 0.61 (95 % CI = 0.53-0.69). Thus, the prevalence of MRSA in hedgehogs is not statistically significantly associated with sex or cause of death, nor does it vary significantly among regions, or between urban and rural habitats.

Detection of MRSA in hedgehog rehabilitators

All hedgehog rehabilitators reported contact with hedgehogs, either on a daily ($n = 3$), weekly ($n = 6$), monthly ($n = 3$), or less than monthly ($n = 4$) basis. However, the nostril and throat samples from the hedgehog rehabilitators showed no growth of MRSA.

Antimicrobial susceptibility testing of the detected MRSA

All MRSA isolates were susceptible to all tested antimicrobials except the β -lactams.

Discussion

Our study confirms a high prevalence of *mecC*-MRSA isolates in Danish hedgehogs (61 %), similar to the 64 % prevalence found in Sweden (Bengtsson et al. 2017). The larger sample size of hedgehogs as well as the inclusion of hedgehogs that died in the wild in our study, strongly suggests that hedgehogs are a natural reservoir of *mecC*-MRSA. Furthermore, our results indicate that the *mecC*-MRSA detected in our study is adapted to animals due to the lack of the *scn*-gene, which is a marker for human adapted *S. aureus* (Sung et al. 2008).

Other natural reservoirs than hedgehogs may exist, but it appears that European hedgehogs have a considerably higher prevalence of *mecC*-MRSA than other mammals. In comparison, Gomez et al. (2014) found a 2 % prevalence of *mecC*-MRSA in 101 faecal samples from six small mammal species in Spain, and a 16.9 % prevalence in nasal swabs from 65 farmed red deer in Southern Spain (Gomez et al. 2015).

Smith & Marples (1965) and Bengtsson et al. (2017) speculated that there could be a fitness advantage for *S. aureus* to become methicillin-resistant due to the presence of dermatophytes in hedgehogs producing penicillin-like substances. This hypothesis seems reasonable, but further investigations are needed to provide any firm conclusions.

The subpopulations of Danish hedgehog are, to varying degrees, geographically isolated. This isolation is reflected by the differences in *spa*-types found in our study. As expected, the effects of this isolation were most pronounced on the smaller islands, e.g. Lolland and Falster (*spa*-type: t1048). The diversity of *spa*-types was higher in Jutland south of the Limfjord than on Zealand, which could indicate mixing with German hedgehog populations in Jutland south of the Limfjord. Interestingly, the three predominating *spa*-types reported from Southern Sweden (t843, t978, t3391) by Bengtsson et al. (2017) also dominated in samples from Zealand.

The subsample of 188 individuals used in this study was stratified by region to ensure coverage across the entire country. However, we emphasize that only a few of the collected hedgehogs were representing the north-western and southern parts of Jutland, due to difficulties with establishing collection stations there. Furthermore, our collection does not reflect the geographical hedgehog distribution in Denmark, since the collection of animals may have been influenced by the human population density and their commitment to the project.

We found a higher prevalence of *mecC*-MRSA in males than in females and, although this was not statistically significant, it is an interesting observation, because males likely have closer and more frequent contact with other individuals than females. This is both due to their promiscuous mating behaviour and due to the fights with other males, particularly during the

mating season, where the males tend to physically fight off other males competing for the favour of the female (Morris 2014). In addition, home ranges of males are generally larger than those of the females (Morris 2014), which would additionally mean that they are more likely to encounter more conspecifics than the females. Smith (1965) also found a higher prevalence of *S. aureus* in males (69 %) than females (40 %).

Hedgehogs receiving care at wildlife rehabilitation centres may be more prone to MRSA acquisition due to their immunocompromised state, being housed closely together and receiving antibiotic treatments. However, we found no statistically significant difference in MRSA prevalence in hedgehogs dying at rehabilitation centres compared to other causes of death, indicating that hedgehogs can be carriers of *mecC*-MRSA regardless of their health status.

Increased population density leads to a greater disease transmission risk (Bradley & Altizer 2007) and, therefore, one might expect a positive association between *mecC*-MRSA and hedgehog population density. Hedgehogs are progressively inhabiting human environments, for instance suburban residential gardens and villages in rural areas, as opposed to agricultural land (Hubert et al. 2011; Pettett et al. 2017; van de Poel et al. 2015). Previous research in the UK has furthermore indicated a greater decline in hedgehog densities in rural areas than (sub)urban areas (Williams et al. 2018), so we expected a higher prevalence of MRSA positive individuals due to the higher population densities in urban and suburban habitats. However, we found no such difference.

We found no evidence for an association between MRSA prevalence and habitat type, region, cause of death, or sex. The high prevalence of MRSA detected in hedgehogs all over Denmark, strongly suggests that hedgehogs are natural reservoirs of *mecC*-MRSA.

A previous case of transmission of *mecC*-MRSA from hedgehogs to humans has been recorded in the National MRSA Register in Denmark, but none of the tested hedgehog rehabilitators tested positive for MRSA, indicating that zoonotic transmission of *mecC*-MRSA from hedgehogs to humans may occur only very rarely. It should however be emphasized that the human samples were collected in late November, which was approximately one month after the release of the last rehabilitated hedgehogs. Prolonged colonization with *mecC*-MRSA seems therefore not to have been established in these workers. Typing of *mecC*-MRSA from livestock indicates that zoonotic transmissions do occasionally occur, thus human cases may not get *mecC*-MRSA directly from hedgehogs but, rather, indirectly via livestock and pets. This speculation is supported by typing of the *mecC*-MRSA isolates from hedgehogs showing

the same *spa*-types as reported before in both humans and livestock (Angen et al. 2017b; Eriksson et al. 2013; Petersen et al. 2013a).

Conclusion

In conclusion, this nationwide study confirms a high prevalence of *mecC*-MRSA in Danish hedgehogs, which could serve as a natural reservoir for this type of MRSA. Other wild reservoirs may exist, but previous studies have only reported sporadic findings in other species (e.g. (Gomez et al. 2014)). Furthermore, this study found no sign of zoonotic transmission of *mecC*-MRSA from hedgehogs to wildlife rehabilitators caring for hedgehogs.

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Supporting information

Table 1. Reclassification of CORINE land cover habitat classes.

CORINE		
code	Label	Reclassified
111	Continuous urban fabric	urban
112	Discontinuous urban fabric	urban
121	Industrial or commercial units	urban
122	Road and rail networks and associated land	urban
123	Port areas	urban
124	Airports	urban
131	Mineral extraction sites	urban
132	Dump sites	urban
133	Construction sites	urban
141	Green urban areas	urban
142	Sport and leisure facilities	urban
211	Non-irrigated arable land	rural
222	Fruit trees and berry plantations	rural
231	Pastures	rural
242	Complex cultivation patterns	rural
243	Land principally occupied by agriculture	rural
311	Broad-leaved forest	other
312	Coniferous forest	other
313	Mixed forest	other
321	Natural grasslands	other
322	Moors and heathland	other
324	Transitional woodland-shrub	other
331	Beaches	other
411	Inland marshes	other
412	Peat bogs	other
421	Salt marshes	other
423	Intertidal flats	other
512	Water bodies	other

S1 Table. CORINE land cover habitat classes found within hedgehog habitat, and how these were reclassified in either “urban”, “rural” or “other”.

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