Catalysing a fruitful future

INTERNSHIP REPORT

A PHYLOGENETIC AND ECOLOGICAL STUDY OF SONGBIRDS EVOLUTIONARY DIVERGENCE:

PHYLOGENETIC STUDY OF THE DIVERGENCE OF MASCARENE MARTENS POPULATIONS

&

MATERNAL AND HABITAT QUALITY EFFECT IN THE REPRODUCTIVE SUCCESS OF GREAT TITS

Irina DELAMARE

From 25/01/2021 To 28/05/2021

Supervisors:

Benjamin Warren (1st internship) & **Clotilde Biard** (2nd internship)

Acknowledgments

Firstly, I would like to communicate my special thanks of gratitude to Dr. Benjamin Warren, lecturer, and manager of the MNHN bird collection, supervisor of my internship on swallows phylogeny; and Dr. Clotilde Biard, lecturer at Sorbonne University, supervisor of my internship on bleu tits and great tits. They both offered me an amazing experience where I was able to discover two facets of birds' biology, focusing respectively on phylogeny and ecology. The time spent working with them helped me in my professional orientation, in the quest to understand my preferences and my limits. Thanks to them, I now have a better understanding of the different fields around bird research.

I would like to bring a special light to the fact that the internship with Clotilde Biard was my first ever field internship and the fulfillment of an old dream. So thank you again for giving me this opportunity.

Secondly, I want to thank every intern I worked with during both internships. We made a really great team and we stuck together in this particularly difficult and isolated year for students. I discovered with them an entirely new way to do research by collaborating with peers that I never experimented within any of my previous experiences. In addition to that, I was able to get familiar with their professional orientation choices and learn a lot about the different BEE masters (Biodiversity, Ecology, and Evolution) from these students, which is the degree I am applying to.

A PHYLOGENETIC AND ECOLOGICAL STUDY OF SONGBIRDS EVOLUTIONARY DIVERGENCE

Abstract

To assess the way passerine populations diverge from one another, I focussed on two case studies approaching intraspecific divergence in both a genetic and phenotypic way. On the one hand, I evaluated the relationship, genetic background, and gene flow between Mascarene martens (*Phedina borbonica*) populations from the Mascarene archipelago and Madagascar in the form of a population genetic study. On the other hand, I looked at the impact of habitat quality and maternal effect on the reproductive success of Great tits (*Parus major*). Both these studies help gauge the impact of our society on the environment and help bring a better understanding of biodiversity in order to preserve ecosystems on the verge of extinction.

Firstly I brought clarity to the phylogeny of Mascarene martens from the Mascarene archipelago and Madagascar by reconstructing the phylogeny of swift based on the HKY genetic distance model using selected nuclear and mitochondrial genes. Mascarene martens sequences were extracted from samples gathered during years of field research, specific nuclear and mitochondrial genes were amplified using polymerase chain reaction (PCR) and were then sequenced.
Secondly, I followed the nesting development of Great tits in rural and urban areas to measure adult female birds' body mass and take feather samples from female birds' bellies in the field for later spectrophotometer analysis. Feather samples were used to assess carotenoid chroma content in the female bird plumage. I used a generalized linear model following a Poisson regression to test two different models explaining reproductive success. The first one hypothesizes that the body mass of female tits explains reproductive success in urban and rural habitats. The second one hypothesizes that habitat, carotenoid chroma, and the interaction of habitat on carotenoid chroma explain reproductive success.

Mascarene martens are a monophyletic and sustained species. There is no gene flow between Mascarene martens from Madagascar, la Réunion, and Mauritius. There does not appear to be gene migration within Madagascar, however, within la Réunion and Mauritius, there is lots of genetic flow. One explanation for the lack of genetic flow in Madagascar relies on the size of the island that could prevent populations living in different habitats from interbreeding. There is no common population of Madagascar and the Mascarene with a genetic exchange, but what appears to be isolated populations.
As expected, the fitness of urban populations of Great tits is lower than that of rural populations. Habitat influences females' great tits body mass, but not yellow belly feathers color. There is no correlation between female tits body mass and color. The body mass of female tits does not explain reproductive success in urban and rural habitats. The interaction of habitat on carotenoid chroma does not explain reproductive success; similarly, carotenoid chroma does not explain reproductive success either. However, habitat explains reproductive success in a statistically significant way. Urban habitat offers a lower reproductive success than rural habitat, and maternal effect does not affect reproductive success.

Passerine populations diverge from one another for multiple reasons. From my two case studies, I isolated a few causes of divergence such as the environment (habitat differences, geographical isolation, environment harshness), bird behavior, and urbanization.

Keywords: Divergent evolution, Urbanization, Phylogeny, Swift, Great tits, Reproductive Success

ÉTUDE PHYLOGÉNÉTIQUE ET ÉCOLOGIQUE DE LA DIVERGENCE ÉVOLUTIVE DES PASSEREAUX

Préambule

Pour évaluer la façon dont les populations de passereaux divergent les unes des autres, je me suis concentré sur deux études de cas abordant la divergence intraspécifique d'une manière à la fois génétique et phénotypique. D'une part, j'ai évalué la relation, le patrimoine génétique et le flux génétique entre les populations de Martinet des Mascareignes (*Phedina borbonica*) de l'archipel des Mascareignes et de Madagascar sous la forme d'une étude génétique des populations. D'autre part, j'ai regardé l'impact de la qualité de l'habitat et de l'effet maternel sur le succès reproducteur des Mésanges charbonnières (*Parus major*). Ces deux études permettent de mesurer l'impact de notre société sur l'environnement et d'apporter une meilleure compréhension de la biodiversité afin de préserver des écosystèmes en voie d'extinction.

Tout d'abord, j'ai apporté des éclaircissements sur la phylogénie des Martinet des Mascareignes de l'archipel des Mascareignes et de Madagascar en reconstruisant la phylogénie des martinet sur la base du modèle de distance génétique HKY en utilisant une sélection de gènes nucléaires et mitochondriaux. Des séquences génétique des Martinet des Mascareignes ont été extraites d'échantillons collectés au cours d'années de recherche sur le terrain, des gènes spécifiques nucléaires et mitochondriaux ont été amplifiés par amplification en chaîne par polymérase (PCR) puis ont été séquencés.
Ensuite, j'ai suivi le développement de la nidification des Mésanges charbonnières dans les zones rurales et urbaines pour mesurer la masse corporelle des oiseaux femelles adultes et prélever des échantillons de plumes sur le ventre des oiseaux femelles pour une analyse au spectrophotomètre ultérieure. Des échantillons de plumes ont été utilisés pour évaluer la teneur en chrominance des caroténoïdes dans le plumage des oiseaux femelles. J'ai utilisé un modèle linéaire généralisé suivant une régression de Poisson pour tester deux modèles différents expliquant le succès de reproduction. Le premier émet l'hypothèse que la masse corporelle des mésanges femelles explique le succès de reproduction dans les habitats urbains et ruraux. Le second émet l'hypothèse que l'habitat, la saturation des caroténoïdes et l'interaction de l'habitat sur la saturation des caroténoïdes expliquent le succès de la reproduction.

Le Martinet des Mascareignes est une espèce monophylétique et soutenue. Il n'y a pas de flux génétique entre les Martinet des Mascareignes de Madagascar, de la Réunion et de Maurice. Il ne semble pas y avoir de migration de gènes à Madagascar, cependant, à la Réunion et à Maurice, il y a beaucoup de flux génétiques. Une explication du manque de flux génétique à Madagascar repose sur la taille de l'île qui pourrait empêcher les populations vivant dans des habitats différents de se reproduire ensemble. Il n'y a pas de population commune de Madagascar et des Mascareignes avec un échange génétique, mais ce qui semble être des populations isolées.
Comme prévu, la fitness des populations urbaines de mésanges charbonnières est inférieure à celle des populations rurales. L'habitat influence la masse corporelle des mésanges charbonnières femelles, mais pas la couleur jaune des plumes du ventre. Il n'y a pas de corrélation entre la masse corporelle et la couleur des mésanges charbonnières femelles. La masse corporelle des mésanges femelles n'explique pas le succès de reproduction dans les habitats urbains et ruraux. L'interaction de l'habitat sur la saturation des caroténoïdes n'explique pas le succès reproducteur ; de même, la saturation des caroténoïdes n'explique pas non plus le succès reproducteur. Cependant, l'habitat explique le succès de reproduction d'une manière statistiquement significative. L'habitat urbain offre un succès de reproduction inférieur à celui de l'habitat rural, et l'effet maternel n'affecte pas le succès de reproduction.

Les populations de passereaux divergent les unes des autres pour de multiples raisons. À partir de mes deux études de cas, j'ai isolé quelques causes de divergence telles que l'environnement (différences d'habitat, isolement géographique, dureté de l'environnement), le comportement des oiseaux et l'urbanisation.

Mots clés: Évolution divergente, Urbanisation, Phylogénie, Martinet, Mésange charbonnière, Succès reproducteur

Graphical abstract

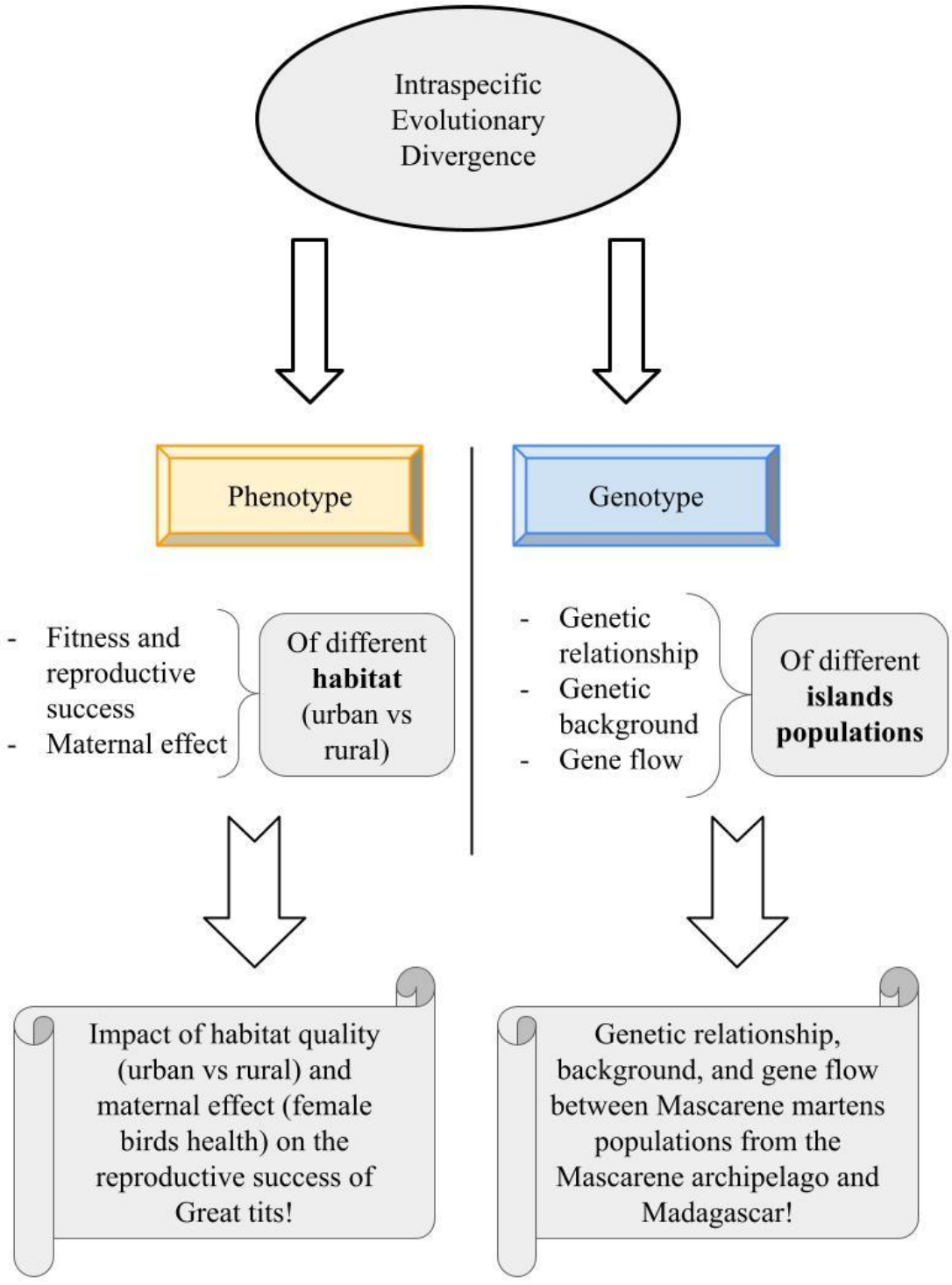

Table of content

Introduction

Birds are warm-blooded vertebrates that diverged from theropod dinosaurs 100 million years ago, during the Late Cretaceous period (1,2). Their reduced size, their ability to fly, and their remarkably agile beaks seem to have contributed to a very successful new body plan allowing them to fill previously unoccupied ecological niches in the ecosystem (2,3). This led birds to evolve much more rapidly than other dinosaurs at the time (3,4), and to represent nowadays an amazing diversity of roughly 18 000 species worldwide, populating all 7 continents, thriving in many different environments, and filling various ecological niches (5). There are nearly 3 times more species of birds than there are species of mammals today (roughly 6 400 mammal species) (6), and about half of them belong to the Passeriformes order, also known as songbirds.

Divergent evolution is the accumulation of genetic and phenotypic differences between closely related populations of the same species. Intraspecific divergence is explained by environmental changes leading to speciation such as allopatric (geographic isolation) or peripatric (peripheral isolation). Although many divergent events occur due to the environment, in some cases, the divergence can be impacted by behavior. For example, passerines have a particularly high rate of anatomical evolution at the organismal level supposedly driven by behavior rather than environmental changes (7). This type of divergent evolution is called sympatric speciation; it occurs when multiple populations of the same species diverge (in the case of songbirds, due to behavioral changes) until they can no longer interbreed despite the fact that they live in the same geographical location.

Divergent evolution doesn't always end up as speciation, in some cases it allows intraspecies diversity. This is particularly true in harsh environments, as environmental harshness is positively correlated with intraspecific divergence in mammals and birds (8). A harsh environment offers lower primary productivity, decreased rainfall, and more variable and unpredictable temperatures compared to the tropics. In these environments, we observe greater subspecies richness than in tropical environments, leading to greater intraspecific divergence in regions with lower biodiversity. Environmental harshness is positively correlated with subspecies richness, as it offers faster rates of evolution, a greater likelihood of qualitative variation occurrence, and more opportunities for divergence (8). The more diverse the pool of individuals, the more diverse the new generation will be, allowing them to adapt efficiently to the harsh fast-evolving environment.

To assess an individual, of a specified genotype or phenotype, the average contribution to the gene pool of the next generation, we use a quantitative representation of natural and sexual selection known as fitness. Fitness refers in evolutionary biology and ecology to the combination of reproductive success and survival and reflects how well an organism is adapted to its environment. The phenotype of an individual is expressed by its genotype and the surrounding environment. One example of environment-induced changes is maternal effects. Maternal effects are defined by the role of the environment and genotype of the mother in determining the phenotype of an organism. Maternal effects can be genetic, where the mother supplies messenger RNA or proteins to the egg that result in phenotypic changes expected from the genotype of the mother, not from the individual's own genotype. However, maternal effects can also be independent of genotype and caused by the maternal environment, sometimes controlling the size, sex, or behavior of the offspring. These phenotypic changes can result in an increase of individual fitness. Furthermore, the maternal effect seems to play an important role in the adaptation of species to a fast-evolving environment (9).

So what rules the intraspecific evolutionary divergence of passerines that allow them to keep evolving in such a diverse way? In other words, how do songbird populations diverge from one another?

To answer this question, I will approach intraspecific divergence in a genetic and phenotypic way, permitting a double understanding of the subject due to the use of two different approaches. By looking at both the evolution of a species and the main factor for its evolution (for example the environment) to study evolutionary divergence, we obtain a complete understanding of the subject. On the one hand, I carried out a population genetic study of the phylogenetic divergence of Mascarene martin populations' to assess the relationship, genetic background, and gene flow between populations from the Mascarene archipelago (Reunion, Mauritius, and Rodrigues) and Madagascar. On the other hand, I performed an evolutionary ecology study of phenotypic divergence supposedly brought by the ecological differences between rural and urban Great tits populations. A focus will be set on diversity within the populations and global health to answer the question: is the fitness of rural populations of Great tits higher than that of urban populations? In other words, how the reproductive success of Great tits is affected by the habitat and the maternal effect?

The impact of our society and way of life on the environment and biodiversity around us is fairly disastrous. Rapid climate change, pollution, and habitat destruction, which we are begetting, do not leave enough time for most species to adapt. A better understanding of biodiversity could help preserve many ecosystems that are on the verge of extinction. Understanding how populations have evolved in the past can help us predict whether these populations are currently in the process of disappearing for a conservation notice. Studying the evolution of populations would predict how urbanization, climate change, and environmental changes could impact them in the near future.

Studying the phylogenetic divergence of swallow populations could help better understand the systematics of different island populations for conservation purposes. In the long term, the projects aim to link together genetic with abundance data (ecological data informing conservation priorities) to add a time-scale to Species Abundance Distribution (SAD). The SAD (10) is a fundamental pattern in ecology and exhibits that worldwide, few species are abundant and many are rare. Adding a time scale to the SAD would help better understand the factors that determine which species are most susceptible to environmental changes and at-risk of undergoing declines towards eventual extinction. To sum up, it would help bring clarity to the role of evolutionary history in determining this fundamental distribution.

Studying the ecological divergence of European tits in urban and rural areas allows mapping of the differences and the impact of urbanity on species fitness. By monitoring European tits in the field year after year, we can observe the impact of gentrification and urbanization on the health and diversity of rural and urban populations of tits. But that is not all. Because birds are an excellent barometer to monitor the environment's wellbeing (11,12), this study brings together crucial information that could help mankind better understand its impact on biodiversity, and hopefully find solutions to preserve the ecosystems and their inhabitants.

To assess the relationship and gene flow between Mascarene Martin (*Phedina borbonica*) populations from the Mascarene archipelago and Madagascar, I will first reconstruct the phylogeny of the Hirundinidae family. Hirundinidae are passerine birds, also known as songbirds just like

tits, found all over the world and encompassing swallows, martins, swifts, and saw-wings. The family Hirundinidae consists of two subfamilies, Pseudochelidoninae (river martins) and Hirundininae (typical swallows) (13). Despite the name Mascarene Martin, this species, that I am studying, belongs to the Hirundininae and not the Pseudochelidoninae. All *Phedina* species are sister groups, meaning that the Phedina genre is monophyletic (13).

We have a pretty clear idea of the phylogeny of Hirundinidae based on three genes (ND2, FIB, cytb) (13). However, the phylogeny of *Phedina borbonica* and the intraspecific divergence of this species between different islands has never been looked at. In order to assess that study, I focused on ND2, and MUSK nuclear genes, and the FIB mitochondrial gene, already present in the Hirundinidae family and commonly used for phylogenetic reconstruction (13–15). I took inspiration from previous publications on the subject (13–15) to select the pairs of primers needed to amplify each gene region.

Passerines are suffering from a loss of evolutionary uniqueness and taxonomic diversity in urban areas leading to a global homogenization of bird communities and a decrease in urban birds' fitness (16,17). The reduction of species richness and evolutionary distinctiveness worldwide support the assumption that the expansion of urban areas reduces biodiversity (16). Phylogenetic study assesses those observations and showcases a diminution of avian genetic diversity. Phylogeny is a good proxy to species rarity and can be used to classify and prioritize species needing protection for conservation plans (18). Because high intraspecific diversity increases species fitness and survival chances when confronted with perturbations (19), urban populations would be prone to disappearing. So a diverse population is a hint to a thriving population.

However, genetic diversity is not the only factor to consider while looking at populations' fitness. Health is a good benchmark to see if populations are at threat of extinction. A diverse diet results in healthy specimens, and health can be measured following morphometric measures such as the weight and size of the bird. Indeed, growth is useful to follow nutritional development as a poor diet is associated with poor health and delayed growth in chicks (20). As a matter of fact, the amount of food and care that is given to chicks affects their adult size and weight (21), so it affects their health. So morphometric measurements such as the weight can help identify how healthy, and well-fed a population is.

Habitat quality also affects population fitness. Urbanization is known to decrease the breeding capacity of birds, meaning that fewer nestlings will reach adulthood (22). Rural chicks are taller, heavier, and more colorful, so have better health, than urban chicks (22). Furthermore, in poor parkland habitats, Great tits chicks show higher physiological stress generated by food-related stressors than in high-quality forest habitats, meaning that habitat quality affects nestlings' growth conditions (23).

Coloration is another good benchmark for diet availability. Carotenoids are antioxidant pigments responsible for the yellow color in Great tits and their plasma concentration is positively correlated to immune system and growth (24–26). The carotenoid-based coloration is also used to signal healthy individuals in developing chicks and adults that create a selection in pair matching and parental investment (22,27,28). Indeed, mating with an ill individual can decrease the reproductive success (in the case the ill specimen dies) and fitness of offspring (bad genotype). Similarly, parental investment into an ill nesting with a low survival rate could decrease the survival rate and food supply of healthy nestlings, thus decreasing the fitness of the entire brood. Carotenoids have both a physiological function (immunity and growth) and a signaling function (signal how healthy an individual is). They can not be synthesized and therefore required to be acquired via a bird's diet (25). They are proof of birds' wellbeing as the richer

in carotenoid the diet of a bird is, the more healthy the bird is (increased body mass and size, increased immunity) and the brighter the yellow belly feathers are (24). So carotenoid-based feathers allow a better understanding of individual fitness. To reflect the carotenoid content of feathers, we calculated carotenoid chroma, a variable used to quantify yellow carotenoid-based coloration in birds (29). Carotenoid chroma assesses the difference between the maximal (plateau at a wavelength above 500 nm) and minimal (wavelengths of maximum absorbance of carotenoids, between 445 and 455 nm) reflectance in the visible part of the spectrum (computed as (R700-R450)/R700) (22,29,30). A low R450 value corresponds to enriched carotenoid in feathers. The more carotenoid an individual will ingest, the more the spectrum will be chromatic. Carotenoid poor feathers will have a high R450 value and a relatively flat spectrum. The bigger the carotenoid chroma number is, the more carotenoids there will be in the feather. Feather reflectance is the effectiveness of the plumage in reflecting radiant energy.

In addition, genetic maternal effects affecting carotenoid content in the yolk, and post-hatching maternal effects relating to treatment might also explain the differences in nestling size and feather color (25). Indeed, higher carotenoid availability influences maternal effect by increasing carotenoid deposition in eggs laid by Great tits females, and positively affects in chicks the development of phenotypic traits associated with fitness (25). Carotenoid rich diet is not available at the same rate during all the breeding season, and females that are able to obtain more carotenoids during the laying period will increase their offspring's fitness, which is another example of maternal effect providing offsprings a better surviving chance to reach adulthood (25). Furthermore, good parental care providing chicks with carotenoid-rich food increases general offspring fitness, and more precisely enhances tarsi length and immune system development (24,25).

To sum up, habitat quality (urban or rural) affects diet quality and availability, which affects specimens' health and size, which affects their fitness and the one of their offspring (maternal effect). Female health impacts the care they bring to their young, so it is a maternal effect. However, female health has not yet been assessed in comparison to habitat quality and reproductive success, though the fitness of rural Great tits is already known to be higher than the one of urban tits (22). We can evaluate the body condition of a specimen by looking at the coloration of its belly feathers, and morphometric measures such as its weight. Reproductive success is measured by the number of nestlings successfully fledged.

Firstly I brought clarity to the phylogeny of swift species *Phedina borbonica* from the Mascarene archipelago and Madagascar in order to determine the conservation priority of the species. To do so, I extracted DNA from samples gathered during years of field research, amplified specific nuclear and mitochondrial genes by PCR, and sent the successful amplification for sequencing. I then compared the sequences to other sequences of different species and reconstructed a phylogeny of swift based on selected nuclear and mitochondrial genes.

Secondly, I followed nesting and chick development of Great tits (*Parus major*) in rural and urban areas to assess how female health affects reproductive success in those populations. I measured adult female birds' weight in the field. I identified the bird's sex and took feather samples from female birds belly for spectrophotometer analysis to assess carotenoid content in the plumage. From this data, I conducted an analysis of the number of nestlings taking off from the nest (representing reproductive success), female body mass and carotenoid-based yellow coloration (health of the female accounting for maternal effect), and habitat quality (urban versus rural habitat).

Motivation for this internship!

My true calling is to become a researcher in biology and work in the field of bird study, whether it is in morphofunctional biology, systematics, or evolutionary ecology. This internship was a perfect occasion for me to discover two of those fields, evolutionary biology, and evolutionary ecology. I was lucky to find two incredible internships allowing me to learn in a rewarding and challenging environment, next to other students and researchers. It is the perfect setting for a young scientist wannabe researcher who wishes to work and evolve in an innovative world using birds as her principal object of study. Those activities were really a bliss that helped me go through those difficult and isolated times of curfew and lockdown. One major motivation was to make a choice between two fields of research, ecology and systematic, between whom I was unsure of my preferences. I successfully explored these two areas of study and now have a better idea toward which I would like to focus to continue my professional career.

Context and Background

I worked with Dr. Benjamin Warren at the Muséum national d'Histoire naturelle de Paris. I worked in the Ornithology department of the Institute of Systematic, Evolution, and Biodiversity (ISYEB) UMR, also known as UMR 7205. The lab belongs to the CNRS (Centre national de la recherche scientifique), the MNHN (Muséum national d'Histoire naturelle), SU (Sorbonne University), and "l'École pratique des hautes études et de l'Université des Antilles" aims to study the origin of biodiversity, the diversification of species and the establishment of communities in relation to the evolution of taxa in time and space. Dr. Benjamin Warren works on a major community-scale ornithological project that links evolutionary biology with ecology (and island biogeography), specifically focussed on the avifaunas of the Mascarene archipelago (Reunion, Mauritius, and Rodrigues) and Madagascar. The projects aim to better understand the systematics of different island populations for conservation. Abundance data have also been collected during years of field research and gives information on conservation priorities. In the long term, the idea is to link together the abundance and genetic data to add a time-scale to species abundance distributions using an individual-based ecologically-neutral model and approximate Bayesian Computation. The major aim of this project is to better understand the role of evolutionary history in determining this fundamental distribution, and factors that determine which species are most susceptible to environmental changes, and therefore to undergoing declines towards eventual extinction.

To do so, Dr. Benjamin Warren is mapping the gene pool of multiple species endemic of the Mascarene archipelago and Madagascar, in order to generate phylogenetic data, and reconstruct dated phylogenetic trees for bird species that have not yet been covered. This is exactly the work I conducted during my internship on the Mascarene swallow also known as *Phedina borbonica* species. Another intern of Dr. Warren worked on Mascarene swiftlet, also known as *Aerodramus francicus,* parallelly of me.

My second internship was with Dr. Clotilde Biard at Sorbonne University. She works at the Institute of Ecology and Environmental Sciences (IEES) which is a UMR common to the CNRS, Sorbonne University, l'Institut de recherche pour le développement (IRD), l'Institut national de la recherche agronomique (INRAE), l'Université de Paris, and Université Paris Est Créteil. The institute's goal is to analyze the organization, functioning, and evolution of ecological and environmental systems in order to make modelizations aiming to better understand the environment. The iEES is committed to understand and predict in order to act on habitat destruction and loss of ecological diversity. It covers a wide range of skills from population dynamics and biodiversity, community ecology and ecosystems, microbial ecology, evolutionary ecology, and the process of adaptation to the environment.

Dr. Clotilde Biard belongs to the Evolutionary Biology department of iEES, in the Evolutionary Ecophysiology (EPE) team. Her research focuses on the study of adaptation to environmental variations in the context of global changes induced by humans, like climate warming and rapid urbanization. She aims her research on maternal effects in birds, particularly great and blue tits. Every year, she takes in a team of interns to keep track of the nesting of great and blue tits. She collects a wide range of data from Foljuif (rural) and multiple parks in Paris and Rueil Malmaison. All nestlings and adults are banded for identification. I was part of this team of interns this year and participated in gathering all that data.

Material and Methods

Internship I: Phylogeny of *Phedina borbonica* estimated from nuclear and mitochondrial DNA sequences

To conduct a phylogenetic study of populations of *Phedina borbonica* from different islands, I first extracted DNA from samples gathered by Dr. Warren during years of field research. Then, I performed an amplification of the extracted sequences by polymerase chain reaction (PCR). I worked on sequencing three different genes, ND2 gene, FIB gene, and MUSK gene, on 26 different specimens of *Phedina borbonica*. I used respectfully H6313 or HTrpC with L5215 primers to sequence the ND2 gene, FibBi7L with FibBi7U or FibRipF with FibRipR for FIB gene, and MUSK-I3F with MUSK-I3R or MuskF and MuskR for MUSK gene. I performed electrophoresis to identify how good my amplifications were in order to decide which sequences I will send for sequencing.

Extracting DNA:

The first step of DNA extraction is to have a sample from which to extract DNA. It can be blood samples, tissues, or even feathers. I had the chance to experiment with 26 samples of all types discussed above. A specific protocol exists for all the different types, however, I used a broad protocol made for both tissue and blood samples extraction in order to facilitate the experimentation process. Also, I noticed after testing blood and tissue specific protocols that they did not work in any noticeably better way than the broad protocol. So I decided to stick with the broad protocol.

Notes for Tissue and Blood sample extraction:

- Perform all centrifugation steps at room temperature (15-25C)
- Redissolve any precipitates in Buffer AL and ATL
- Buffer AW1 and Buffer AW2 are dissolved in ethanol

Broad Protocol for Tissue and Blood sample extraction:

- Cut tissue (< 25mg) into small pieces, and place in a 1.5mL microcentrifuge tube. Add 180microL Buffer ATL. If dealing with blood samples, add 75microL of blood to a 1.5mL microcentrifuge tube and adjust the volume to 200microL with PBS. Add 20microL proteinase K to the 1.5mL microcentrifuge tube containing either blood or tissue samples, mix by vortexing and incubate at 56C until complete lysis (>12h overnight). Vortex occasionally during incubation.
- Vortex 15s then add 200 microL of Buffer AL to the sample. Mix thoroughly by vortexing.
- Add 200microL ethanol (100%). Mix thoroughly by vortexing.
- Pipet the solution into the DNeasy Mini spin column placed in a 2mL collection tube. Centrifugate at 8000rpm for 1 min, then discard the collection tube and flow-through.
- Place the DNeasy Mini spin column in a new 2mL collection tube. Add 500microL Buffer AW1 and centrifugate at 8000rpm for 1 min. Discard collection tube and flow-through.
- Place the DNeasy Mini spin column in a new 2mL collection tube and add 500microL Buffer AW2. Centrifugate at 14000rpm for 3min to dry the DNeasy membrane. Discard collection tube and flow-through.
- Place the DNeasy Mini spin column in a new 2mL microcentrifuge tube and add 200microL Buffer AE onto the membrane. Incubate at room temperature for 1 min, then centrifugate at 8000rpm for 1 min. The solution present in the 2mL microcentrifuge tube is the final solution. Repeat this step to have a better DNA yield.

PCR amplification:

After extracting DNA from tissue samples, I amplify the resulting solution using polymerase chain reaction (PCR). It is a selective way of exponentially amplifying genetic material by using repeated thermal cycling. To do so, we use two primers, a forward primer and a reverse primer, and a solution of nucleotides. I am working on different pairs of sequences belonging to different parts of the genetic code. For example, I work on FIB and MUSK regions, which are nuclear sequences, and the ND2 region, which is a mitochondrial sequence. I am using a multitude of primers to identify which ones amplify each region of genes best. For each primer, I need to test the best temperature of annihilation. To do so I perform gradient PCR where all samples to be amplified are heated at different temperatures to identify which one best fits the primer. The ideal primers and their adequate temperature are assessed during the electrophoresis step.

For the FIB region, I tested FibBi 7U and FibBi 7L pair of primers, and FibRipF and FibRipR pair. None of the two pairs of primers I tested showed concluent results and the amplifications are not good enough to work with. For ND2 region, I tested HTrpc and L5215 pair, and H6313 and L5215 pair. However, this second pair did not show good amplifications so I left it out. For the MUSK region, I tested MuskF and MuskR pair, and Musk-13F and Musk-13R pair. Amplification results I got from both pairs are good, so they are compatible, meaning that I can assemble the results together as they are amplifications of the same region.

Now that I know the primers pairs that amplify correctly, and after getting the adequate temperature of annihilation with the PCR gradient, I am left with amplifying all my 26 samples 3 times (one for each good pair of primers). In order to do that, I need to make a PCR solution (see Figure 1 below to get acquainted with the protocol and the PCR cycle resulting).

MIX

	per tube for a total of 20µL (µL)
H20	10.7
Tampon 10x	2
MgCl2	1.5
dNTP	0.8
Primer F	0.4
Primer R	0.4
Taq QIAGEN	0.2
BSA	1
DMSO	1
ADN	2
	TOTAL 20 µL

PCR Cycle

94 C	2:00	x1
94 C	0:30	
57 C	0:30	x35
72 C	0:30	
72 C	7:00	x1

Figure 1: Protocol of PCR solution and PCR cycle

PCR migration:

To identify if PCR amplification has worked, I performed migrations on an agarose gel, also called electrophoresis. First, I prepare the agarose gel.

Protocol of the agarose gel:

- Add 60 mL of TAE to a becher
- Add 1g of agarose and mix gently
- Put the solution into the microwave until ebullition is reached (careful, the resulting solution is quite hot and need to be handled with care)
- Add 1microL of BET (BET is cancerogenic and must be handle under a fume cupboard)
- Pour the solution on an adapted surface in order to create wells

After letting the gel thicken for about an hour, I placed it in a solution filled with TAE buffer. I mixed 5microL of my amplified DNA solution with drops of loading dye and put it in the wells of the agarose gel. An electrical current of 100V is applied for 25 minutes beginning the migration process in a gel electrophoresis apparatus. The negatively charged DNA migrates toward the positively charged anode. When the migration is over, I observe the results of the electrophoresis using UV light. The loading dye that we mixed earlier with our amplified DNA solution is visible through UV light, giving a visual clue of the migration of the DNA. The flashier the signal will be, the better the amplification (Figure 2 below).

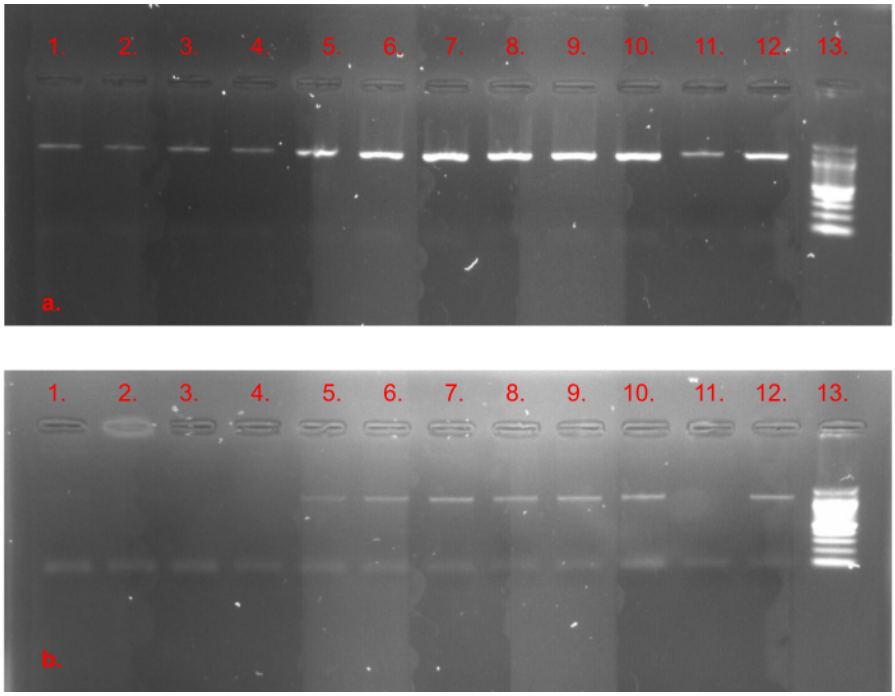

Figure 2: Two electrophoresis of PCR products. a and b wells contain the same DNA samples, but are amplified with different primers, each amplifying sequences of different regions of the nuclear and mitochondrial genome. HTrpC-L5215 pair of primers was tested in a, and FIB7 U-L pair of primers was tested in b. We can clearly see a difference in PCR amplification efficiency and quality between a and b. b electrophoresis shows two visible bands (the one below is a primer dimer), and DNA is not clearly visible for every band (electrophoresis b. 1 to 4 and 11 which wells correspond to blood samples). FIB7 primers did not correctly amplify the DNA sequences of blood samples.

Sequences analysis:

After having identified sequences that had amplified well, I sent them for sequencing by a company working with researchers from the MNHN. The results of the sequencing came back with two elongations, one for each primer (forward and reverse), for each specimen. Not every sequence came back clean from the sequencing, so some of them are not usable. I assembled the two elongations of the acceptable sequences into one sequence. After that, I cleaned the sequence by hand from mistakes made by the algorithm. I generated consensus sequences for each specimen and for each DNA region. I added my sequences (ingroups) to a matrix of sequences of the same region in different species of related martens and willows (outgroups). I was able to create neighbor-joining trees for the two different genes I worked with, putting in comparison my ingroups with the outgroups. It allows me to compare my sequences to other sequences of different specimens, in order to locate each specimen phylogenetically.

However, those trees are not linked to one another, meaning they each propose a phylogenetic relationship between different species by only taking into account one gene or region. Getting a phylogenetic tree that takes into account both genes to understand the evolutionary history and relationships between species is equivalent to taking a specific model and running our matrix through it. The first step to use this method is to combine congruent genes in order to have more data and more accurate results. Congruence in evolutionary biology is the way characters are coherent with one another. Combining congruent genes will allow us to run our model with both the mitochondrial and the nucleic genes of *Phedina borbonica*. What I mean by combining is literally stacking my two sequences belonging to the same specimen one behind the other in order to create a matrix containing all mitochondrial and nuclear sequences. I removed from this matrix all the outgroups (the sequences of other species than *Phedina borbonica*). This work was performed on Geneious software version 5.4.7.

I used Jmodeltest, a program for the statistical selection of models of nucleotide substitution, on CIPRES, a science gateway providing public resources for inference of large phylogenetic trees, to get an evolutionary model for my matrix.

After running the Jmodeltest program, I concluded from the resulting file that regardless of the method used it is always the “HKY + I” substitution model that is supported as best fitting my data. Hasegawa–Kishono–Yano (HKY) model assumes that nucleotides occur at different frequencies, and that transitions and transversions occur at different rates. “+I” refers to invariant sites. Based on the result, I used the HKY genetic distance model to plot a tree built on the Neighbor-Joining method (with 1000 iterations).

Internship II: Maternal effects, habitat quality, reproductive success of Great Tits

In order to test whether the breeding habitat and maternal effects affect the reproductive success of Great tits, the statistical interaction between habitat and maternal effects on reproductive success will be tested by a generalized linear model using an ANCOVA test. I will compare urban habitat with rural habitat, and use female body mass and carotenoid-based yellow breast feather color to measure female quality assessing maternal effect. The number of fledglings is used to assess reproductive success and is a dependent variable, also known as discrete, and thus follows a Poisson distribution.
Data were collected from 2010 to 2019 in l'ile de France where Dr. Biard has implemented approximately 330 nests in the past years. The nests are disposed in multiple parks in Paris (Jardin des Plantes, Parc de Belleville, Parc des Buttes-Chaumont, Montsouris, Kellerman, le cimetière Montmartre) and Rueil Malmaison (cimetière de Bulvis, Petite Malmaison, Carré Bellemare) for urban area, and for rural area she placed nests near Nemour Saint Pierre in Foljuif forest and La Commanderie forest (near the CNRS' Station Biologique de Foljuif). Nests were inspected regularly to determine laying date, clutch size, incubation date, hatching date, number of hatchlings and unhatched eggs, and number of fledglings. After the first egg was laid, nests were visited once every 2 days until the beginning of incubation. Final clutch size was recorded. Once incubation has started, nests were visited less frequently until the day before the estimated hatching date (incubation date + 14 days), and then visited each day until hatching. Adults were captured using a fishing stick and a ping-pong ball (see Annex 1) and individually bended with Museum aluminum rings. Chicks were captured directly in the nest a few days before the approximate fledging date. Female quality was assessed by measurement of their weight and brightness of their belly feathers. Body mass to the nearest 0.25 g was measured with a spring balance. Six to eight yellow feathers samples from the belly were plucked, and stored in the dark in plastic bags for later analysis.
Yellow breast feathers' color was analyzed using a spectrometer. From the 8 feather samples per specimen, two batches of 4 are made at random and stacked together. 4 measurements were made per batch. Then, we averaged the 8 measurements for each color variable. To reflect the carotenoid content of feathers, we calculated carotenoid chroma that assesses the difference between the maximal (plateau at a wavelength above 500 nm) and minimal (wavelengths of maximum absorbance of carotenoids, between 445 and 455 nm) reflectance in the visible part of the spectrum (computed as (R700-R450)/R700) The bigger the carotenoid chroma number is, the more carotenoids there will be in the feather.

Data analysis:

I am going to test habitat effects on female tits body mass and color using a sample composed of 116 urban female great tits and 313 rural female great tits from France. To do that, I used a generalized linear model following a Poisson regression with log as linking function using an ANCOVA test in R version 3.6.3. To perform this test, the interaction between each variable needs to be elucidated by preliminary analysis. I used a correlation test to look for a correlation between female tits body mass and color, and a T-test to compare female body mass in rural and urban habitats, and female color in rural and urban habitats. To perform a T-test, we need the two groups of samples to be normally distributed (Shapiro test) and to have equal variances (F test). After preliminary analysis, we concluded on 2 different models to be tested using a Poisson regression model. The first one hypothesizes that the body mass of female tits explains reproductive success in urban and rural habitats. The second one hypothesizes that habitat, carotenoid chroma, and the interaction of habitat on carotenoid chroma explain reproductive success.

My program (R notebook) is available on my GitHub account here:
https://github.com/irinade/S6_Internship_on_Great_tits- . The dataset I used was not made public so is lacking on GitHub.

Results

Internship I: Phylogeny of *Phedina borbonica* estimated from nuclear and mitochondrial DNA sequences

I used the HKY genetic distance model to plot trees built on the Neighbor-Joining method (with 1000 iterations). I can conclude based on my results that 100% of *Phedina borbonica* specimens are grouped together in the phylogenetic reconstruction of the Hirundinidae family (see Figure 4 below). However, less than 70% (63.8% to be exact) of phylogenetic reconstructions link together specimens from the Mascarene and Madagascar (Figure 3).

88.3% of the time, all individuals from the Mascarene (Mauritius and la Reunion) are grouped together, except for M96 specimens. (Figure 3) This is showcased by looking at the bottom node of the tree (Figure 3). The M96 specimen behaves weirdly and is surprisingly located close to specimens from Madagascar and not Mauritius. This specimen was also weirdly located in previous builds (not even placed in *Phedina borbonica* species in some cases) and is probably fallacious and to be considered as so.

84% of the time, specimens from Mauritius are grouped together, and 75.1% of the time, specimens from La Réunion are assembled together. (Figure 3) Specimens from Madagascar, on the opposite, are not grouped together and no nodes are apparent in 87% of simulations.

Surprisingly, *Phedina brazza* is not phylogenetically close to *Phedina borbonica.* (Figure 4)

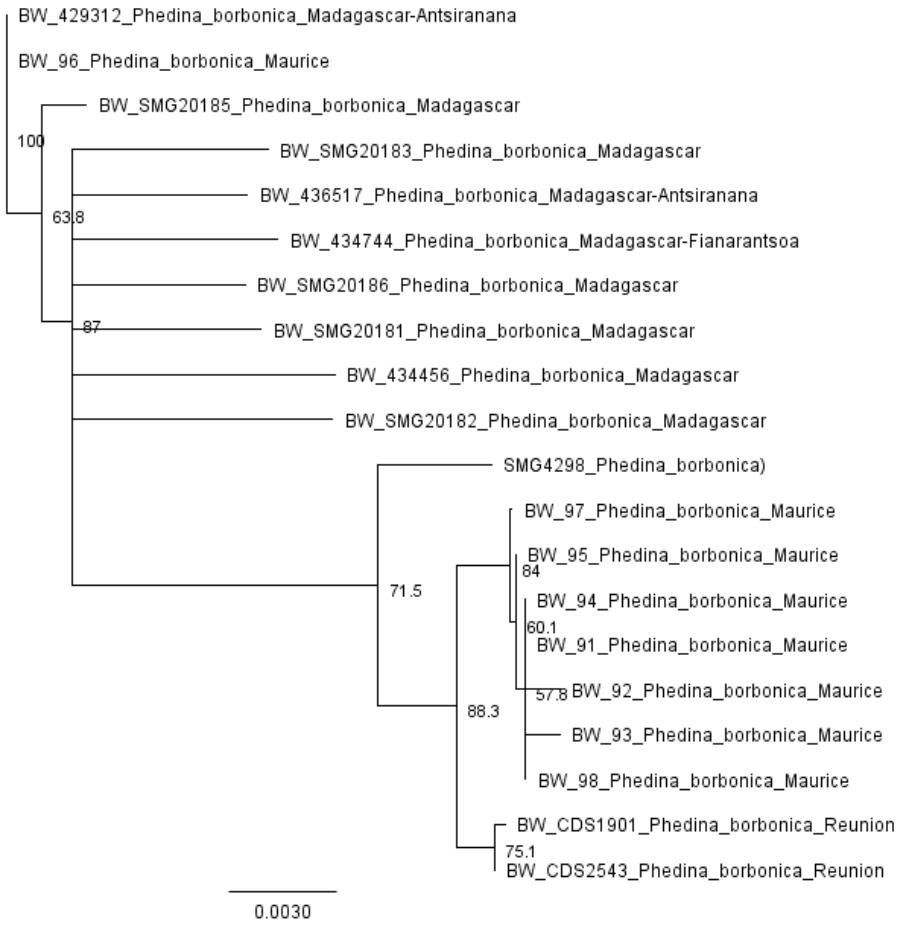

Figure 3: Neighbor-Joining consensus and concatenated (take into account both ND2 and MUSK genes) tree of *Phedina borbonica* from the Mascarene archipelago and Madagascar based on the HKY genetic distance model with 1000 iterations.

Figure 4: Neighbor-Joining consensus and concatenated (take into account both ND2 and MUSK genes) tree of Hirundinidae family based on the HKY genetic distance model with 1000 iterations.

We are going to use three coefficients to analyze our result, the t-statistic, the z-score, and the p-value. The t-statistic is used in a t-test to determine whether to support or reject the null hypothesis. The z-score value informs on the standard deviations distance from the mean. If a z-score is equal to 0, it is on the mean. If a z-score is positive, the raw score is higher than the mean average, and vice versa. The p-value informs on the statistical significance of the result. If the p-value is small, the hypothesis should be rejected. A p-value less than 0.05 is statistically significant.

Shapiro test and graphical analysis to test the normality of my samples:

A Shapiro test and a graphical analysis are used to test the normality of two groups of samples. From the output obtained using the Shapiro test, we can assume normality for the coloration (*p-value* = 0.365 > 0.05) but not from the body mass (*p-value* = 0.0006 < 0.05). The p-value of the color dataset is greater than 0.05. Hence, the distribution of the given data is not different from the normal distribution significantly. The p-value of the body mass dataset is lower than 0.05. Hence, the distribution of the given data is significantly different from a normal distribution.

However, this was expected because of the large dataset I am working on. Testing graphically the normality of color and body mass will give a less strict approximation of normality. Graphically, we can assess that both the color and body mass dataset follow a normal distribution because the observed quantiles of the two distributions are well aligned with the theoretical quantiles of the normal law.

Correlation test:

Because my data on color and body mass follows a normal distribution, I looked for a correlation between female tits body mass and color. I am using a Pearson correlation (*p-value* = 0.4814 ; *t* = -0.7046) which measures a linear dependence between two variables (col and bm). It is a parametric correlation test because it depends on the distribution of the data of the variables, which needs to follow a normal distribution. The p-value is approximately 0.48 which is here considered equal to 0.5 (5%), thus the correlation between color and body mass is non-significant. t value, which gives the difference between the two means, is close to zero.

F test to compare variance homogeneity:

An *F test* is used to compare the homogeneity of variances for both female tits body mass in rural and urban habitats (*p-value* = 0.691), and female tits color in rural and urban habitats (*p-value* = 0.2301). Depending on the homogeneity of variances, the *T-test* that will follow will not be the same. P-value for the *F test* of female tits body mass in urban and rural habitats is approximately 0.69 (>0.05). And p-value for the *F test* of female tits carotenoid chroma in urban and rural habitats is approximately 0.23 (>0.05). So there is no significant difference between the variances of body mass and carotenoid chroma in urban and rural habitats. Consequently, we can use the *T-test* and suppose variances equality for both body mass and color.

T-test to compare female body mass in rural and urban habitats, and female color in rural and urban habitats:

P-value for *T-test* of female tits body mass (*p-value* = 5.472e-11 ; *t* = -6.7302) in urban and rural habitat is approximately 0 which is much less than 0.05. We conclude that the mean body mass of female tits in urban habitat is significantly different from that of rural habitats. Because the difference between urban body mass and rural body mass is negative (t<0), we can conclude that the mean body mass of female tits in rural habitat is heavier than in urban habitat. And the p-value for *T-test* of female tits carotenoid chroma in urban and rural habitat (*p-value* = 0.05564 ; *t* = 1.9191) is approximately 0.05. So the mean carotenoid chroma of female tits in urban habitat is significantly no different from that of rural habitat.

This boxplot illustrates what the *T-test* showed, that rural female great tits have on average a higher body mass value than urban ones. (see figure 5 below)

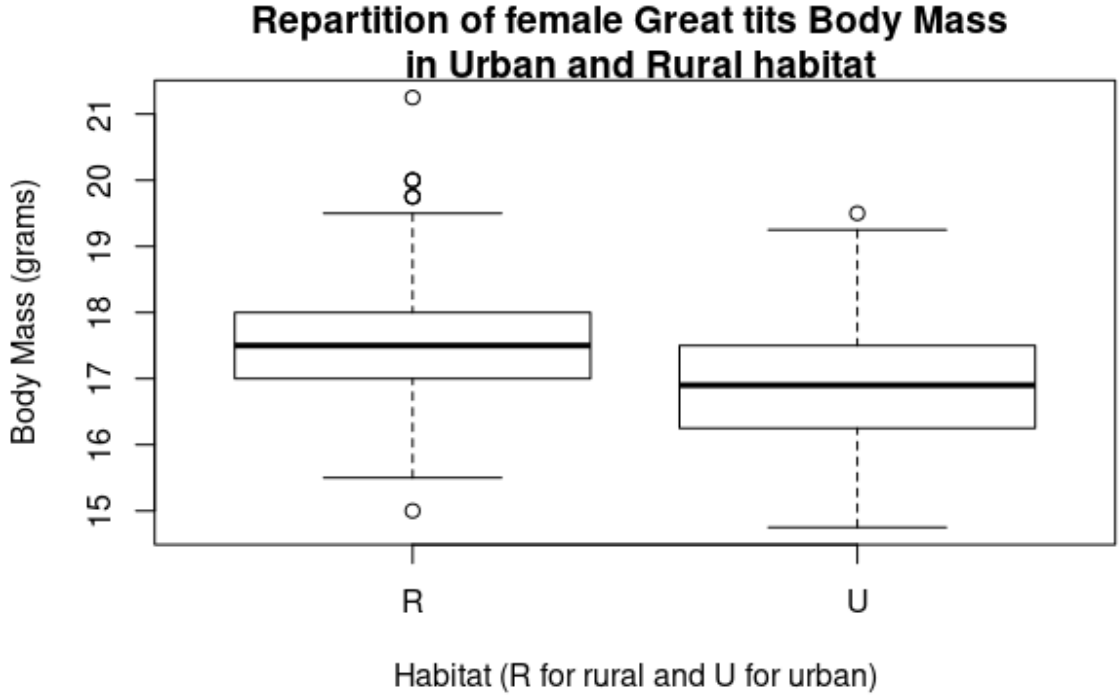

Figure 5: Boxplot showing the repartition of female great tits body mass in grams between urban and rural habitat.

To sum up, habitat influences mass, but not color, and there is no correlation between body mass and color. So there are two models to test by using a generalized linear model following a Poisson regression with a log as a link function.

First, we hypothesize that the body mass of female tits explains reproductive success in urban and rural habitats. (*glmRS <- glm(nPenv ~ Pds, data=dataU, family=poisson())* and *glmRS <- glm(nPenv ~ Pds, data=dataR, family=poisson())* where dataU represent the urban dataset and dataR the rural dataset, RS is the short for Reproductive success, nPenv is the short for the number of fledglings, and Pds refers to the body mass)

Second, we hypothesize that habitat, carotenoid chroma and the interaction of habitat on carotenoid chroma explains reproductive success. (*glmRS <- glm(nPenv ~ Habitat + mCarotChroma +*

Habitat:mCarotChroma, data=data, family=poisson()) where mCarotChroma refers to carotenoid chroma)

To verify the hypothesis that the body mass of female tits explains reproductive success in urban habitat, we need to look at the resulting coefficient (z = -0.28 ; *p-value* = 0.77 > 0.05 ; *degrees of freedom of residual deviance* = 114). P-value of the generalized linear model testing the hypothesis that body mass of female tits explains reproductive success in urban habitat is larger than 0.05, meaning that the relationship is not statistically significant. In rural habitat we observe a similar result (z = -1.09 ; *p-value* = 0.27 > 0.05 ; *degrees of freedom of residual deviance* = 311) with a p-value significantly larger than 0.05. So reproductive success is not explained by female body mass in either urban or rural habitats.

To verify the second hypothesis assessing that habitat, carotenoid chroma, and the interaction of habitat on carotenoid chroma explains reproductive success, we need to take a look at the coefficient of each of these variables independently. First, let us start with the interaction effect of habitat on carotenoid chroma (z = -1.68 ; *p-value* = 0.09 > 0.05 ; *degrees of freedom of residual deviance* = 424). We observe that the p-value is slightly bigger than 0.05, so the interaction of habitat on carotenoid chroma to explain reproductive success is not statistically significant. So we can remove this interaction from our model and run it back again. Afterward let us look at carotenoid chroma (z = 0.304 ; *p-value* = 0.76 > 0.05 ; *degrees of freedom of residual deviance* = 425). We observe that the p-value is significantly larger than 0.05, so carotenoid chroma does not explain reproductive success in a statistically significant way. So we can remove this interaction from our model and run it back again. Now let us look at habitat (coefficients for urban habitat : z = -4.38 ; *p-value* = 1.17e-05 < 0.05 ; *degrees of freedom of residual deviance* = 427). We observe that the p-value is significantly smaller than 0.05, so habitat explains reproductive success in a statistically significant way. Because z-score is negative, the raw score of urban habitat is lower than the mean average. Meaning that urban habitat offers a lower reproductive success than rural habitat.

Conclusion and Perspectives

First, I assessed the relationship, genetic background, and gene flow between populations from the Mascarene archipelago and Madagascar in the form of a population genetic study of the phylogenetic divergence of Mascarene martin (*Phedina borbonica*) populations'. Second, I looked at the impact of habitat (urban and rural) and maternal effect (female health) on reproductive success (number of fledglings) of Great tits (*Parus major*). As expected, the fitness of urban populations of Great tits is lower than that of rural populations, affirming the hypothesis that urbanity decreases populations' intraspecific diversity.

Phedina borbonica is a monophyletic and sustained species because 100% of our specimens are grouped together in the phylogenetic reconstruction of the Hirundinidae family (Figure 4). It means that they are more closely related to one another than to other sequences of different species. There is no interspeciation.

The relationship between *Phedina borbonica* from Madagascar and the Mascarene is not sustained (see Figure 3). This means that there is not a common population of Madagascar and the Mascarene with genetic exchange, but what appears to be isolated populations. A sustained relationship is obtained when more than 69% of phylogenetic reconstructions simulate similarly the placement of a specimen, which is not the case here. Moreover, specimens from Mauritius seem more closely related to one another than the ones from Madagascar based on the nodes' sustenance.

The relationship between different specimens of *Phedina borbonica* from Madagascar suggests that there is no Madagascar population because they are not sustained. This is represented by not having a node regrouping all specimens from Madagascar in the tree.

In the Mascarene however, we clearly observe a sustained relationship between Mauritius and La Réunion populations. We observe lots of genetic flow within Mauritius and la Reunion, but not much between the two islands. This is called reciprocal monophyly. These two populations constitute sister lineages but do not interbreed. Both Mauritius and La Reunion are monophyletic.

We can hypothesize that one specimen from Madagascar has immigrated to the Mascarene and is the common ancestor of all *Phedina borbonica* from the Mascarene. Many genetic mixity and flow have come out from within Madagascar specimens, based on the absence of nodes regrouping Madagascar specimens (Figure 3). Ancestral gene copies do not combine into a common ancestral copy in specimens from Madagascar because the evolutionary divergence is far too recent to divide into groups. This is called incomplete lineage sorting. Oppositely, specimens from the Mascarene have few genetic mixity.

To sum up, there is no gene flow between *Phedina borbonica* from Madagascar, la Réunion, and Mauritius. There does not appear to be gene migration within Madagascar, however, within la Réunion and Mauritius, there is lots of genetic flow. Because environmental harshness is positively correlated with intraspecific divergence in birds (8), and because there is more intraspecific diversity of Mascarene martens population in the Mascareigne, does it mean that the Mascareigne offers a harsher environment than Madagascar? This questioning could be interesting to follow up on.

One explanation for the lack of genetic flow in Madagascar is that Madagascar is a huge island with a lot of diverse and different habitats and many populations could live there without interbreeding. My study is probably suffering from a lack of data. With more samples from Madagascar, I would expect to find different genetic populations from Madagascar that do not breed with one another. Morphological divergence can be driven more by habitat differences than geographic isolation (31). Parapatry selection may weigh more heavily than geographic isolation in causing adaptive divergence in morphology, explaining the lack of one interbreeding population in Madagascar (31). Another hypothesis leads to the idea of sympatric speciation, where population divergences are impacted by behavior rather than geographical isolation (7).

What is unexpected is that *Phedina Brazza*, another species belonging to the Phedina genre, is not closely related phylogenetically to *Phedina borbonica*. (see Figure 4) *Phedina Brazza* is far from *Phedina borbonica* on the phylogenetic tree, leading to the suggestion that the Phedina genre is polyphyletic. However, the *Phedina brazza* sequence we used in our matrix came from "Phylogeny of swallows (Aves: Hirundinidae) estimated from nuclear and mitochondrial DNA sequences" paper from Frederick H Sheldon and Linda A Whittingham (13). This sequence is astonishingly short compared to other sequences in the matrix, which must explain the strange observation we see in Figure 4.

For further phylogenetic analysis, Dr. Benjamin Warren recommends using the parameters selected by the BIC method that he already uses in his publications. The next step would be to conduct a phylogenetic analysis, however, as my internship was approaching its end I was not able to do so. It was expected as Dr. Warren warned me that three months were required to properly complete my internship subject, however, he could only propose a two-month internship. Dr. Warren recommends trying a Bayesian phylogenetic analysis with Beast2 software to go further.

The distribution of carotenoid chroma and body mass of female great tits (*Parus major*) follows a normal distribution as graphically we observe the alignment of theoretical quantiles and obtained quantiles. There is no significant difference between the variances of body mass and carotenoid chroma in urban and rural habitats. There is a homogeneity of variances for both female tits body mass in rural and urban habitats, and female tits color in rural and urban habitats.

There is no statistically significant correlation between female tits body mass and carotenoid chroma. So the yellow-breasted feather color and the body mass of female tits are not correlated. This result is surprising as carotenoid is supplied by the bird's diet (25), and a good diet results in large healthy birds (21). Furthermore, carotenoid plasma concentration is positively correlated to nestlings' growth (24–26). Finding no correlation between adult female tits body mass and carotenoid chroma can be explained by the effect of time on each variable. Body mass is partially determined during nestlings' growth and depends on parents' involvement and food supply. Carotenoid chroma is not set during nestling growth and change during the lifetime of tits. The absence of correlation between adult female color and body mass might be explained by parental investment, which differs from female investment into themself. Because parents are responsible for nestlings' care and food supply, which is a kind of post-hatching maternal effect, and because parents' investment has an impact on adult bird's size and health, the adult bird's weight is partially independent of the bird's capacity to feed himself. However, the carotenoid chroma content of adult birds is set by the capacity of the birds to look for rich carotenoid food. So body mass of adult birds depends on parental investments (maternal effects), and yellow-breasted coloration

depends on the adult bird's own capacity at finding food. From this assumption, we propose that nestlings' color and body mass are correlated. This hypothesis might be interesting to look into.

The mean body mass of female tits in urban habitat is significantly different from that of rural habitats (see figure 5). Rural female tits are on average heavier than urban females. Because body mass is a benchmark to assess the health of a specimen and the food availability in his environment, we can assume that on average rural female tits are healthier than urban's ones. Because the diet and care given to chicks affect their adult weight and health (21), we can assume that urban populations do not have access to as rich of a diet and as much care as rural populations, resulting in lighter adult females. So urbanity's way of life does permanently affect the body conditions of female great tits.

On the other hand, the mean carotenoid chroma of female tits in urban habitat is significantly no different from that of rural habitat. So on average, there is no difference in yellow-breasted feather coloration of urban and rural female great tits. Because carotenoids can only be found in the diet, and have a positive effect on birds' physiological function on immune system and growth, and a signaling function on the healthiness of an individual, we expected to see a statistical difference between rural and urban habitat. The lack of difference might be linked to the fact that adult birds are responsible for their own nutrition. Because carotenoid chroma is not set during nestling growth and change during the lifetime of tits, there is no way to assess the carotenoid chroma of chicks by looking at adults. Because carotenoids affect nestlings' growth (24–26), and because we found that rural birds are on average bigger than urban ones, we can hypothesize that chicks' carotenoid chroma is higher in rural habitat than in urban habitat.

The first hypothesis we tested showcased that the body mass of female tits does not explain reproductive success in urban and rural habitats. As we said before, body mass is a good benchmark for birds' health and diet availability. So female health does not affect reproductive success in urban and rural habitats. So maternal effect does not affect reproductive success, thus offspring fitness.

The second hypothesis showed that the interaction of habitat on carotenoid chroma does not explain reproductive success; similarly, carotenoid chroma does not explain reproductive success either. Habitat influence on yellow-breasted feather color, and yellow-breasted feather color does not affect reproductive success in great tits. However, habitat explains reproductive success in a statistically significant way. And urban habitat offers a lower reproductive success than rural habitat. So female birds living in an urban environment will have on average lower reproductive success than rural birds. These results are in accordance with other studies made on the subject assessing that urbanization decreases the breeding capacity of birds (22).

To sum up, habitat influences females' great tits body mass, but not yellow belly feathers color. Furthermore, there is no correlation between female tits body mass and color. Moreover, urban habitat offers a lower reproductive success than rural habitat, and maternal effect does not affect reproductive success. In other words, the fitness of rural Great tits is higher than that of urban populations.

Multiple reasons push passerines populations to diverge from one another. In the case of Mascarene martens, the environment (habitat differences, geographical isolation, environment harshness) or bird behavior leads to the intraspecific divergence of the different island populations. In the case of Great tits, urbanization is responsible for population divergence and fitness. This report only assessed some reasons responsible for divergence, the top of the iceberg if you want, but there is still so much more topic left unstudied.

References

1. Feduccia A. ‘Big bang’ for tertiary birds? Trends Ecol Evol. 2003 Apr 1;18(4):172–6.

2. Brusatte SL, O’Connor JK, Jarvis ED. The Origin and Diversification of Birds. Curr Biol. 2015 Oct;25(19):R888–98.

3. How Birds Evolved From Dinosaurs [Internet]. Quanta Magazine. [cited 2021 Mar 30]. Available from: https://www.quantamagazine.org/how-birds-evolved-from-dinosaurs-20150602/

4. Brusatte SL, Lloyd GT, Wang SC, Norell MA. Gradual Assembly of Avian Body Plan Culminated in Rapid Rates of Evolution across the Dinosaur-Bird Transition. Curr Biol. 2014 Oct;24(20):2386–92.

5. Barrowclough GF, Cracraft J, Klicka J, Zink RM. How Many Kinds of Birds Are There and Why Does It Matter? PLOS ONE. 2016 Nov 23;11(11):e0166307.

6. Upham N. How many species of mammals are there? J Mammal. 2018 Feb 1;99:1–14.

7. Wyles JS, Wilson AC. Birds, behavior, and anatomical evolution. :4.

8. Botero CA, Dor R, McCain CM, Safran RJ. Environmental harshness is positively correlated with intraspecific divergence in mammals and birds. Mol Ecol. 2014;23(2):259–68.

9. Mousseau T, Fox C. Mousseau TA, Fox CW. The adaptive significance of maternal effects. TREE 13: 403-407. Trends Ecol Evol. 1998 Oct 1;13:403–7.

10. Baldridge E, Harris DJ, Xiao X, White EP. An extensive comparison of species-abundance distribution models. PeerJ [Internet]. 2016 Dec 22 [cited 2021 Apr 16];4. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5183127/

11. Carignan V, Villard M-A. Selecting Indicator Species to Monitor Ecological Integrity: A Review. Environ Monit Assess. 2002 Aug 1;78(1):45–61.

12. Prasad S. ‘Birds are indicators of a healthy and thriving environment.’ The Hindu [Internet]. 2018 May 15 [cited 2021 Jun 4]; Available from: https://www.thehindu.com/news/cities/puducherry/birds-are-indicators-of-a-healthy-and-thriving-environment/article23886055.ece

13. Sheldon FH, Whittingham LA, Moyle RG, Slikas B, Winkler DW. Phylogeny of swallows (Aves: Hirundinidae) estimated from nuclear and mitochondrial DNA sequences. Mol Phylogenet Evol. 2005 Apr;35(1):254–70.

14. Schweizer M, Olsson U, Shirihai H, Huang Q, Leader P, Copete J, et al. Contrasting patterns of diversification in two sister species of martins (Aves: Hirundinidae): The Sand Martin Riparia riparia and the Pale Martin R. diluta. Mol Phylogenet Evol. 2018 Aug 1;125:116–26.

15. Zink RM, Pavlova A, Rohwer S, Drovetski SV. Barn swallows before barns: population histories

and intercontinental colonization. Proc R Soc B Biol Sci. 2006 May 22;273(1591):1245–51.

16. Ibáñez-Álamo JD, Rubio E, Benedetti Y, Morelli F. Global loss of avian evolutionary uniqueness in urban areas. Glob Change Biol. 2017;23(8):2990–8.

17. Vandewoestijne S, Schtickzelle N, Baguette M. Positive correlation between genetic diversity and fitness in a large, well-connected metapopulation. BMC Biol. 2008 Nov 5;6:46.

18. Mace GM. Preserving the Tree of Life. Science. 2003 Jun 13;300(5626):1707–9.

19. Gamfeldt L, Källström B. Increasing intraspecific diversity increases predictability in population survival in the face of perturbations. Oikos. 2007;116(4):700–5.

20. Naef-Daenzer B, Keller LF. The foraging performance of great and blue tits (Parus major and P. caeruleus) in relation to caterpillar development, and its consequences for nestling growth and fledging weight. J Anim Ecol. 1999;68(4):708–18.

21. Björklund M, Westman B. Adaptive advantages of monogamy in the great tit (Parus major): an experimental test of the polygyny threshold model. Anim Behav. 1986 Oct 1;34(5):1436–40.

22. Biard C, Brischoux F, Meillère A, Michaud B, Nivière M, Ruault S, et al. Growing in Cities: An Urban Penalty for Wild Birds? A Study of Phenotypic Differences between Urban and Rural Great Tit Chicks (Parus major). Front Ecol Evol [Internet]. 2017 [cited 2021 Jun 4];5. Available from: https://www.frontiersin.org/articles/10.3389/fevo.2017.00079/full

23. Banbura J, Skwarska J, Banbura M, Gladalski M, Holysz M, Kalinski A, et al. Spatial and Temporal Variation in Heterophil-to-Lymphocyte Ratios of Nestling Passerine Birds: Comparison of Blue Tits and Great Tits. PLOS ONE. 2013 Sep 16;8(9):e74226.

24. Biard C, Surai PF, Møller AP. Carotenoid availability in diet and phenotype of blue and great tit nestlings. J Exp Biol. 2006 Mar 15;209(6):1004–15.

25. Biard C, Surai PF, Møller AP. Effects of carotenoid availability during laying on reproduction in the blue tit. Oecologia. 2005 Jun;144(1):32–44.

26. Saino N, Ferrari R, Romano M, Martinelli R, Møller AP. Experimental manipulation of egg carotenoids affects immunity of barn swallow nestlings. Proc R Soc Lond B Biol Sci. 2003 Dec 7;270(1532):2485–9.

27. Fitze PS, Tschirren B, Richner H. Carotenoid-based colour expression is determined early in nestling life. Oecologia. 2003 Sep 1;137(1):148–52.

28. Saino N, Suffritti C, Martinelli R, Rubolini D, Møller AP. Immune response covaries with corticosterone plasma levels under experimentally stressful conditions in nestling barn swallows (Hirundo rustica). Behav Ecol. 2003 May 1;14(3):318–25.

29. Delhey K, Roberts ML, Peters A. The carotenoid-continuum: carotenoid-based plumage ranges from conspicuous to cryptic and back again. BMC Ecol. 2010 May 26;10:13.

30. Örnborg J, Andersson S, Griffith SC, Sheldon BC. Seasonal changes in a ultraviolet structural colour signal in blue tits, Parus caeruleus. Biol J Linn Soc. 2008 Jun 28;76(2):237–45.

31. Testing alternative mechanisms of evolutionary divergence in an African rain forest passerine bird - Smith - 2005 - Journal of Evolutionary Biology - Wiley Online Library [Internet]. [cited 2021 Jun 7]. Available from: https://onlinelibrary.wiley.com/doi/full/10.1111/j.1420-9101.2004.00825.x

Annex 1

The capture of the adult's Great tits:

- Place the fishing stick 30 to 50m from the nest and prepare the material for banding and morphometry.
- Release the brake, and walk to the nest silently by unwinding the fishing line with the ladder and the ping-pong ball, and 2 catch bags (1 to block the entry of the nest, and one for the captured birds)
- Pass the ball line from the outside to the inside of the door through the nest hole, tie the loop of the ball line to the swivel of the fishing line going to the reel. The ball should hang well below the bottom of the nest box. By pulling sharply, check that the wire slides well and that the ball is blocking the entrance to the nest box. Return to your observation post, and tension the line by rewinding the reel: the line must be sufficiently tight, but not too tight. Apply the reel brake to arm the trap.
- Observe the back and forth movements of the adults feeding the chicks and proceed to capture the 1st adult when they enter the nest.
- Capture the trapped bird and rearm the trap to capture the second bird.