Brief summary of Ph.D. theses

Factors influencing the timing of the bird migration, especially the sex-related differences

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1. Introduction

1.1. Sex identification methods of birds

Defining the sex of birds can be crucial for scientific studies and captive breeding, as well. However, many bird species (and almost all nestlings) can only be sexed via molecular methods outside the breeding period. Universal diagnostic primers that may sex most Neognathae birds are widely used. These usually amplify different sized fragments of a gene on the sex chromosomes (Schmid et al., 1989). In birds, females are the heterogametic sex, and thus have two morphologically different chromosomes (Z, which is common with males, and W, which is present only in females), which help the separation of the fragments. One of the widely used genes for the molecular sexing is the CHD1 (Chromodomain-Helicase DNA Binding Protein1), which has well-noted size difference between CHD1-W and CHD1-Z introns (e.g. Fridolfsson and Ellegren, 1999).

Although the identified primer pairs appear to be universal for most of the Neognathae bird species, earlier studies showed that they do not work with similar efficiency among the species or between sample types. Therefore, it is important to compile a protocol, which contains recommendations to help in the selection of the best possible methods and markers for any given type of sample or species.

1.2. Age- and sex related differences in the patterns of bird migration

Migration is the most complex part of the life of birds, since it is determined by genetic elements, but is also driven by environmental factors. The optimal arrival and stop-over duration time are key elements of the studies on migration because these shape the migration strategy and determine the survival of the individuals as well.

Although the timing of migration is under strong genetic control, in the last decades more studies detected significant changes in migration phenology. For instance, due to the increasing temperature in early spring the optimal arrival date could be moved forward (Ahola et al., 2004; Both et al., 2006). The earlier arrival date is positively correlated with the breeding success, but the weather affects the mortality, so there is a strong selection for the optimal timing. However, the changes in the phenology and the migratoy strategy differ not only among species, but also among age- and sex groups of the same species.

The age-related difference may not be caused only by the inexperience of the juveniles before their first migration. Timing of different parts of the life-cycle (e.g. moult, building fat deposit) could also vary (Newton, 2008) and affect the arrival and departure time. Furthermore, the migration distance from the breeding site could also correlate with the timing (e.g. Morganti et al., 2011).

There are two types of sex-related patterns in the timing of migration: protandry, if the males arrive and migrate earlier and protogyny, if the females migrate earlier (Morbey and Ydenberg, 2001). During spring, protandry was found in most Passerine species. This might be connected to the early occupation of territories to maximize the breeding success. Possible explanations of the phenomenon could be for example the higher speed of migration or higher photosensitivity (Coppack and Pulido, 2009). In contrast, during the autumn migration, fewer differences could be detected and these patterns varied among closely related species and populations as well. Protandry was found in some species, where the males invest less in breeding and can start earlier the preparation for migration (Lehikoinen et al., 2017). Protogyny is much less frequent than protandry, and it occurs in species where the females migrate greater distances from the breeding site (e.g. Catry et al., 2004; Mills, 2005).

However, it is important to note, that the above-discussed patterns and differences are based on the results of studies, which focus on sexually dimorphic species. It would be necessary to analyse the sex-related phenology in monomorphic species as well, otherwise it is possible to overlook significant trends in changes of timing and migration strategy. However, combined analyses of the age- and sex-related differences may be complicated, due to the necessity of molecular sexing in several species after the breeding season.

1.3. Impacts of blood parasites on bird migration

The immune status and condition of the individuals are key elements during migration, which could be affected by endo- and ectoparasites. Migration is a complex, directional and energetically highly demanding action, which can be immune suppressing and therefore increase the susceptibility to infection.

In the acute phase of infection mortality can be higher (e.g. Ilgūnas et al., 2019), and they might change the timing of the surviving individuals. For example, infected individuals need more time to refuel and could depart form a stop-over site later, than an non-infected ones (Hegemann et al., 2018). These differences affect the arrival to the wintering site and thus can have significant impact on migration success and induce carry-over effects on other annual-cycle stages like breeding.

Haemosporidian parasites, due to their potential effects on the whole life cycle of the birds, are the focus of many studies. These blood parasites strongly affect the reproductive success or female mate selection (Lapointe et al., 2012). An increasing number of the studies found that malaria infection had significant impacts during migration as well. For example, infected individuals arrived later and in some species the infected individuals were in worse energetic condition after spring migration (pl. DeGroote et al., 2010; Emmenegger et al.,

2018). However, knowledge about the effects during the autumn migration is still lacking.

2. Aims of the dissertation

In the first part of the dissertation, I tested four frequently used universal bird sexing markers in 13 Neognathae bird orders. I examined two main questions: (1) whether the reliability of these markers differs between various avian orders and (2) whether the type of the tissue samples affects the success of the molecular sex determination.

In the second part, I compared the timing of the autumn migration based on several aspects. My studied species were the Pied Flycatcher (*Ficedula hypoleuca*), the Reed- (*Acrocephalus scirpaceus*)*,* Sedge- (*A. schoenobaenus) and* Marsh Warbler (*A. palustris*) and Robins (*Erithacus rubecula*)*.* My questions and comparisons were categorized as follows:

Age- and sex related patterns in timing of bird migration

(1) Are there any changes in the sex ratio during the autumn migration and in the Pied Flycatcher during the spring migration as well?

(2) What age- or sex-related changes did happen in the timing of spring and autumn migration over the last two decades in the case of Pied Flycatchers?

(3) Is there any difference in the length of the time spent at the stop-over sites between the males and females?

Age- and sex-related patterns in the changes of the biometrical traits during migration

> (4) Are there any age- and sex-related differences in the biometrical traits (e.g. wing length, body mass)?

> (5) What age- or sex-related changes did happen in biometrical traits (wing length, body mass) during the season?

> (6) What age- or sex-related changes did happen in body condition (visible fat deposit) during the season?

In the case of Robins, during the autumn migration I examined (1) whether the overall prevalence of avian malaria parasites differs between age- and sex groups; (2) if there is a relationship between malaria infection status and biometrical traits (wing feather length, body mass) and condition (fat scores) of the individuals, and (3) whether the timing of arrival differs between infected and non-infected individuals.

3. Material and methods

3.1. Field data

A total of 799 samples of more than 60 bird species were provided from more sources (various LIFE projects, annual ringing monitoring programs, Actio Hungarica, breeders). I used 315 feather- and 484 blood samples for comparison.

Data for the study of bird migration were collected at the Ócsa Bird Ringing Station. The ringing station itself is at the edge of

a post-glacial bog with mosaic, heterogeneous vegetation ranging from reedbeds to forests. This area plays an important role as a breeding and stop-over site during migration (Csörgő et al., 2016). The studied species have different migration strategies such as long or short migration distance, using more or less stop-over sites, etc. All selected species are sexually monomorphic, except the Pied Flycatcher. In the case of this species, I used a longterm database containing data collected between 1989-2016 (N_{spring}= 434, N_{autumn}= 2289). In the case of the other species (Reed Warbler, N= 703, Sedge Warbler, N= 683, Marsh Warbler, N= 408 and Robins, N= 1010), I took blood samples from birds captured at Ócsa for molecular sexing over the course of three years. I also prepared blood smears from samples taken from 406 Robins in autumn 2016. These samples served as positive controls during the molecular detection of parasites.

3.2. Molecular methods

DNA was extracted according to tissue type (feather or blood) using DNA kit following the manufacturer's protocol. I had chosen four universal bird sexing markers for testing: P2/P8 (Griffiths et al., 1998), 2550F/2718R (Fridolfsson and Ellengren, 1999), CHD1-i16F/R and CHD1-i9F/R (Suh et al., 2011). Amplified fragments were visualised with UV light, following a 2% agarose gel electrophoresis.

When studying migration patterns, P2/P8 and CHD1-i9F/R primer pairs were used for molecular sexing. For the molecular detection of avian malaria parasites, I used a highly efficient nested polymerase chain reaction (PCR) method (Waldenström et al., 2004). Identification of Haemoproteus and/or Plasmodium infection was based on amplification of a specialized fragment in the cytochrome b gene (Ricklefs and Fallon, 2002).

3.3. Statistical methods

I used generalized linear models (GLM) to study **changes in sex ratio** during autumn migration **in relation to arrival time**. I defined arrival time as the 'day of the year' when the given bird was first caught at the study site (Harnos et al., 2015). The dependent variables were day of the year, year, age and day of the year*age and year*age interaction.

I calculated **the stop-over duration** based on the time elapsed between the first and last captures at the study site. The data was separated by sex and in the case of Robins by age as well.

In the case of the Pied Flycatcher, I used linear mixed effects (LME) models to detect **changes in the timing of migration**. In addition to year, I included sex, age and their interaction in the models. I analysed the effects of weather parameters in separate models that did not include year as a fixed factor, instead year was a random factor in all models.

Concerning the **changes in biometrical traits in relation to arrival time** I used three variables. First, I applied a GLM to estimate the changes in odds to have visible fat deposit (0= no, 1= yes) during the season in all species except the Pied Flycatcher. The response variable was the fat score (0/1), the independent variblaes were year, age, sex, day of the year, and the day of the year*sex, day of the year*age and sex*age interactions. To study the **changes in body mass and wing length during the migration** I applied linear models, where the independent variables were the year, age, sex, day of the year, and the day of the year*sex, day of the year*age and sex*age interactions. In the case of the Pied Flycather due the longterm data series I was able to apply LME models, where the year was used as fixed and random factor as well. I selected the best model in all case using Akaike's information criterion.

I calculated the **prevalence in all age and sex categories** with Sterne's methods (Sterne, 1954) and compared them with Fisher-test. Due to the small sample size in the infected adult group ($N_{infected}$= 10), I discarded all adult birds from the later models. I compared the **average wing length and body mass of the infected and non-infected individuals** using two-way ANOVA with multiple comparison tests. I assessed the **relationship between infection status and fat scores** using GLM. GLM was also used to study the **changes in the infected/non-infected ratio during autumn** migration in relation to arrival time and sex. To study the possible

differences in the **average arrival time of the individuals** I used GLM. Independent variables were sex and infection status and their interaction.

Prevalence was calculated and compared with Quantitative Parasitology 3.0 (Reiczigel and Rózsa, 2005), all other analyses were performed using R version 3.4.2 (R Development Core Team, 2017).

4. Results and Conclusions

4.1. Feasibility of universal sexing markers in birds

When comparing the various tissue types the greater sized feathers and blood samples stored in alcohol proved to be the most reliable for molecular sexing. 21% of the small sized feathers gave no detectable results. The marker CHD1-i9 did not work in 14.5% of the feather samples. Such error occurred only in 4% of blood samples.

In conclusion the four tested primer pairs can be regarded as easy-to-use tools for molecular sexing in birds, but with some consideration. The marker P2/P8 needed the longest electrophoresis time, but did not yield visible sex-specific bands even after 120 minutes in nine species. In the case of the CHD1-i16, aspecific bands that can make the correct sex assignment difficult were common, but using a "touchdown" step during thePCR these extra bands can be avoided. Further, no sex-specific bands were detected with 2550F/2718R in seven species.

Based on my experience I suggest to consider the following, before planning molecular sexing of birds:

1. When choosing feathers as samples, it is important to use greater and freshly moulted ones.

2. If there is no previous data about the reliability of the sexing markers available for the studied species, using CHD1-i9 is not recommended. Especially if only small or degraded feather samples are available. If possible, it is recommended to start with the simultaneous testing of at least two markers, and also the duration of the gel electrophoresis should be at the first time at least 60 minutes.

3. In order to test the reliability of the selected primers, it is highly suggested to collect samples from individuals of known sex.

4.2. Factors affecting the timing of migration
Age- and sex related patterns

Male Pied Flycatchers were migrating through our study site earlier than the females, and the rate of protandry increased over the study period. Moreover, our study provided evidence that the arrival times of males correlate with local air temperature. However, the arrival time of females did not change over the studied periods and showed no correlation with temperature. This pattern corresponds to the changes observed in the other studied populations, that the warmer spring may

provide optimal conditions for earlier-arriving males. The differences in the arrival date of sexes may also be influenced as different selection pressures, which will be stronger in males than in females. Helm et al. (2019) found that in the last decades some elements of the life cycle of males moved forward, however, no remarkable changes were found in females. The mechanism behind the males' earlier arrival might be caused by flying faster or starting the migration earlier. Ouwehand et al. (2017) found that there was no sex-related difference in the migration speed in spring, however, males departed from the wintering grounds earlier than females.

Contrary to this pattern the onset of autumn migration of Pied Flycatcher remained the same for all sex- and age groups. Consequently, the timing of breeding and the time before migration probably did not change, similarly to what was found in some European breeding populations of the species (e.g. Ahola et al. 2004). Helm et al. (2019) found that after the dispersion period, the juveniles showed later the same "migration-restlessness" as the females, however the timing of postjuvenile moulting, which is a key element during the time before departure, did not changed.

In adult Acrocephalus species I detected protandry in autumn, meaning the ratio of males decreased during the season. This might be caused by the unequal breeding investment of sexes, in these species. Females typically invest more in breeding than

males, so they are able to start migration later (Leisler et al., 2002; Lehikoinen et al., 2017). Furthermore, in all species the sex ratio was female-skewed in all years (except in juvenile Reed Warbler in 2014), however there were no sex-related differences in the stop-over durations (number of days between the first and last capture) and in the recapture ratio.

In Robins there was no significant sex-related difference in the arrival time. It is possible, that the differences in the phenology of sexes vanished because of the mixing of the cross-migrating populations from different breeding sites. For instance, at the end of migration, the proportion of newly captured males may decline because dominant individuals tend to winter in the northern area, or arrive earlier at our study area and did not migrate further.

Biometrical traits

According to the results of body mass data analysis within one migration season the heavier individuals arrived later to the study site in male Pied Flycatcher in spring. However, in females and in both sexes in autumn no sex-related patterns could be found. In spring there was no relationship between the wing lenght and the arrival time, however in autumn the longer winged individuals migrated later in all age- and sex groups. The spring patterns could be explained by preparing for the male-male competition for the breeding territories. The males migrating earlier might tend to by-pass stop-over sites before

arriving at the breeding grounds, meaning they arrive with lower fuel deposits to Europe. In contrast, the later migrating males could achieve advantage in competition, if they are in better condition. Probably this trade-off between arrival time and condition did not play role in the migratory startegy of females. However, in autumn it is possible that Pied Flycatchers do not fuel their deposit in Carpathian-basin or just at a lower rate.

In Acrocephalus warblers I got similar patterns, the individuals with greater body mass, but with lower chance to have fat deposits arrived earlier and their wing length was shorter on average (except juvenile Sedge Warbler, where the body mass correlate positively with the arrival date). There were no sex-related differences in these patterns. It may strengthen the fact that the earlier migration of males might not be driven by the better condition at the beginning of the migration, but possibly by the earlier departure or faster speed of migration. The longer winged individuals arrived later during the season in both age- and sex groups. This correlation is the same as it was found based on longterm database at this study site (Pásztory-Kovács, 2013). They suggested that the detected pattern could be explained by (1) the later arrival of the longer winged males, or (2) the longer winged individuals from the northern breeding population. In addition to my results, the second hypothesis should be more likely, and in the case of Reed Warbler, this means that individuals using two different migration paths differ in average wing length and timing.

Robins have shorter wings on average, but with better body condition they migrate later in both age groups. Dominance might also shape this pattern, the subdominant individuals, with smaller body size on average, could arrive later at the study site and start to increase their fuel deposits in the late period of the migration season.

To sum up, these results draw attention to interesting patterns in the migration phenology of the long-distance migrants. The earlier studies suggest that the increasing level of sexual dimorphism cause greater differences in the arrival date of males and females. In spring, this means an increasing proportion of protandry (Saino et al., 2010), but in autumn it can cause rather a protogyny (Lehikoinen et al., 2017). Based on my results, although the male Acrocephalus warblers have longer wings and larger body mass than females, they migrate earlier. Furthermore, the different reproductive strategies contradict the similar migration phenology. The males probably have territories on stop-over and wintering sites as well, and the selection pressures could be higher to arrive early on the wintering ground. On the other hand, the habitat circumstances during preparation of migration could also cause differences in timing. In seasonal forests food availability declined much stronger than in less seasonal marshes, so the male and the later migrating female Acrocephalus warblers have similar feasiblity to achieve the optimal condition for migration. However, in the forests, where food abundance during the

breeding season is also not constant, the optimal period to refuel is more scarce and males and females might not be able to shift their departure date.

4.3. Effects of avian-malaria infection during the autumn migration of Robins

The overall prevalence of blood parasites was 14.9% in the sample and did not differ significantly according to the age or sex groups. I found no significant differences in wing length, body mass and fat accumulation in relation to infection status, which would suggest little effects of malaria parasites on the condition of the individuals during autumn migration. However, the ratio of the infected individuals increased during the season, and the infected juveniles arrived on average 5 days later than the non-infected.

Previous studies showed that the prevalence of avian malaria parasites had temporary changes, which is partly determined by the life cycle of the parasites, by the host's sensibility to infection, and by the limitation of detection technics (Valkiūnas, 2005). Prevalence had a peak in the spring, so most of the studies focused on this period. Older individuals were frequently found to be more infected than the 1-year-old ones, however, the sex-related differences vary among the species (e.g. Wood et al., 2007; Szöllősi et al., 2016). On the other hand, there can be multiple reasons, why I did not find any differences:

1. The definition of the age groups is different during autumn: in spring the two groups are the 1-year-old and adult individuals, which have more time to get infected like the juveniles hatched in the year of capture.

2. Parasitaemia might show different dynamics in the different age groups during autumn: the majority of the juveniles probably still carry infections with relatively high parasitaemia, on the other hand, the parasites had already started to disappear from the blood of the adult birds. Therefore, a part of the infections went undetected in adults at the time of blood sampling. And as a result, we found a similar prevalence in the two age groups.

3. There was no difference in the probability of infection between males and females during autumn migration, perhaps because the difference in the behaviour, which affect the chance to become infected, disappear after the breeding periods.

During migration good body condition (body mass, visible fat deposit) is a key element in the survival and in the timing of arrival and departure. Earlier studies found that infection in the acute phase increased the mortality (e.g. Ilgūnas et al., 2019) and showed negative effects on actual body condition of the individuals (e.g. Valkiūnas et al., 2006). These findings suggest that parasite infections might affect the timing and length of migration in different bird species (e.g. Emmenegger et al.,

2018). To sum up, during autumn migration the arrival time of juvenile Robins tended to correlate with their infection status independently of their actual condition. Infected individuals arrived later at the stop-over site in Hungary, which might caused an increased stop-over duration time (like in Hegemann et al., 2018). To improve our understanding of the impacts of these parasites on the migratory phenology of their hosts, further studies are clearly needed. More inter- and intra-populational comparative studies, especially the simultaneous sampling of the host populations along their migratory routes, on their breeding and wintering sites would be needed to understand the annual dynamics and impacts of infection.

5. Results new to science

Applicability of universal CHD1 sexing markers in various bird species

1. In CHD1-i16, aspecific bands making sexing diagnosis difficult were usually present, therefore the insertion of a "touchdown" step in the PCR protocol is recommended in case of the studied species.

2. Opposite of the earlier published results in some species, the detection the Z- and W-linked bands of P2/P8 was possible on agarose. Only longer electrophoresis time was needed.

3. In the studied four owl species the CHD1-W chromosome-linked fragments were longer than 1000 bp in the case of the 2550F/2718R and CHD1-i16 primers. Therefore, these two markers are appropriate for sex identification on an agarose gel as well.

Comparing age- and sex-related patterns of migration between sexually dimorphic and monomorphic species

4. The difference in the timing of spring migration of male and female Pied Flycatchers increased in the last two decades at our study site. Only arrival times of passage migrant males correlated with local daily temperature variables. During autumn, there was no difference in the arrival time of males and females. In the three Acrocephalus warbler species the males

migrated earlier. In the migration patterns of the Robins no sex-related patterns could be found.

5. There were no sex-related differences in the recapture rate and stop-over duration in all studied species. Only female Marsh Warbler stayed longer at our study site than males.

6. Only age-related differences were found in the changes of the average body mass during the migration. In adult and juvenile Robins, adult Reed and Marsh Warbler decline was observed, but in the adult Sedge Warbler increase was found. In juveniles the average body mass either increased or did not change during the migration. The rate of the individuals with visible fat deposits increased during the autumn migratory season in all species. In Acrocephalus species, the average wing length of captured individuals increased during the migration period. In Robins individuals with longer wings on average arrived earlier, but the difference was marginal.

Effects of avian-malaria infection during the autumn migration of Robins

7. The overall prevalence of blood parasites was 14.9% at our study site, but there were no age- or sex-related differences in the prevalence. Infected individuals did not differ either in their biometrical traits or in their fat accumulation scores, however, infected juveniles tended to arrive later at the stop-over site.

6. Publication related to dissertation

1. Full text publications in peer-reviewed journals with an impact factor assigned

Harnos A., **Ágh N.**, Kovács Sz., Lang Zs., Csörgő T.: **Increasing protandry in the spring migration of the Pied Flycatcher (*Ficedula hypoleuca*) in Central Europe**, Journal of Ornithology, 156. 543-546, 2015, doi: 10.1007/s10336-014-1148-3 (**IF$_{2015}$:** 1,419)

Ágh N., Kovács Sz., Nemesházi E., Szabó K.: **Univerzális, ivarhatározáshoz használt CHD1 markerek alkalmazhatósága különböző madárrendekben**, Magyar Állatorvosok Lapja, 140. 47-59, 2018 (**IF$_{2018}$**: 0,143)

Ágh N., Piross I.S., Majoros G., Csörgő T., Szöllősi E.: **Malaria infection status of European Robins seems to associate with timing of autumn migration but not with actual condition**, Parasitology, 146(6). 814-820, 2019, doi: 10.1017/S0031182018002184 (**IF$_{2019}$**: 2,456)

2. Oral and poster presentations at international and Hungarian conferences

Ágh N., Kovács Sz., Harnos A., Csörgő T.: **Nádiposzáta fajok őszi vonulásának kor- és ivarfüggő mintázatai**, IX. Magyar Természetvédelmi Biológia Konferencia, Szeged, 2014 (poster)

Ágh N., Kovács Sz., Harnos A., Csörgő T.: **Nádiposzáta fajok őszi vonulásának kor- és ivarfüggő mintázatai**, Akadémiai

Beszámolók: Parazitológia, állattan, halkórtan szekció, Budapest, 2015 (oral)

Kovács Sz., Harnos A., **Ágh N.**, Lang Zs., Csörgő T.: **Increasing protandry in the spring migration of the Pied Flycatcher (*Ficedula hypoleuca*) in Hungary**. 10th European Ornithologist's Union, Badajoz, 2015 (poster)

Ágh N., Kovács Sz., Harnos A., Csörgő T.: **Sex and age-related autumn migration of 3 Acrocephalus species in Hungary**, 4th Student Conference on Conservation Science, Cambridge, 2015 (poster)

Ágh N., Kovács Sz., Harnos A., Csörgő T.: **A vörösbegy őszi vonulási stratégiáját meghatározó tényezők vizsgálata, különös tekintettel a kor és ivarcsoportok közötti különbségekre**, Akadémiai Beszámolók: Parazitológia, állattan, halkórtan szekció, Budapest, 2016 (oral)

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Ágh N., Kovács Sz., Fehérvári P., Harnos A., Csörgő T.: **Exceptional protandry in autumn migration timing of three closely related *Acrocephalus* species**, 3rd Student Conference on Conservation Science Hungary, Tihany, 2017 (poster)

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