Spolne razlike u morfologiji i tempiranju selidbe vrsta zviždak Phylloscopus collybita (Vieillot, 1817) i brezov zviždak Phylloscopus trochilus (Linnaeus, 1758) u sjevernoj Poljskoj

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Sex differences in morphology and migration timing of Chiffchaff *Phylloscopus collybita* and Willow Warbler *Phylloscopus trochilus* in northern Poland

Master thesis
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This thesis was produced at the Bird Migration Research Station of University of Gdańsk, under guidance of dr. hab Magdalena Remisiewicz and assoc. prof. dr. sc. Ivana Maguire. Thesis was submitted to be evaluated by the Biology department of Faculty of Science at the University of Zagreb, with the purpose of obtaining a title of "Master of Science".

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SEX DIFFERENCES IN MORPHOLOGY AND MIGRATION TIMING OF CHIFFCHAFF *PHYLLOSCOPUS COLLYBITA* AND WILLOW WARBLER *PHYLLOSCOPUS TROCHILUS* IN NORTHERN POLAND

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Chiffchaff (*Phylloscopus collybita*) and Willow Warbler (*Phylloscopus trochilus*) are two sympatric bird species breeding across most of Europe and a part of Asia. During autumn and spring migration to Africa and southern parts of Europe, north-European populations of these species migrate along the coasts of the Baltic Sea. Both species are monomorphic in plumage, which impedes any research of potential sex differences in their migration behaviour.

I collected and analysed blood samples of individuals of both species captured during their migration through the Baltic coast in northern Poland, to determine their sex genetically, by DNA from blood and feather samples. Based on the measurements of DNA-sexed individuals, I developed criteria for morphological sexing of each species. I applied these criteria to sex unsampled birds captured at the same ringing site during several migration seasons in the past, to identify sex differences in their migration timing and the effects of weather on migration. Differential migration in the form of protandry was revealed in both species. The timing of males' passage varied considerably from year to year in Chiffchaff, but less so in Willow Warbler, pointing to a more rigid migration schedule in the latter species. Additionally, temperature appears to influence migration timing and choice of stopover in Chiffchaffs.

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SPOLNE RAZLIKE U MORFOLOGIJI I TEMPIRANJU SELIDBE VRSTE ZVIŽDAK *PHYLLOSCOPUS COLLYBITA* (Vieillot, 1817) I BREZOV ZVIŽDAK *PHYLLOSCOPUS TROCHILUS* (Linnaeus, 1758) U SJEVERNOJ POLJSKOJ

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Zviždak (*Phylloscopus collybita*) i brezov zviždak (*Phylloscopus trochilus*) dvije su srodne vrste malih pjevica, čiji areal rasprostranjenosti obuhvaća većinu europskog kontinenta i dio Azije. Tijekom proljetne i jesenske selidbe sjevernoeuropske populacije obje vrste koriste dio selidbenog puta koji prati obalu Baltičkog mora. S obzirom da spolove ovih vrsta nije moguće razlikovati na temelju obojenosti njihova perja, spolno specifične selidbene strategije ovih populacija do sada nisu detaljno istraživane.

U sklopu ovog istraživanja sakupljala sam uzorke krvi zviždaka i brezovih zviždaka za vrijeme njihove selidbe kroz sjevernu Poljsku, kako bih im genetički odredila spol. Dobivene podatke o spolu sam iskoristila za morfološke usporedbe muških i ženskih jedinki, te uspostavljanje kriterija za morfološko određivanje spola. Pomoću tako uspostavljenog kriterija sam zatim odredila spol jedinkama ulovljenim tijekom ranijih proljetnih sezona migracije. Analiza tempiranja selidbe pokazala je da mužjaci obje vrste stižu na područje gniježđenja prije ženki. Raspored selidbe zviždka podložniji je utjecaju temperature i zamjetno više varira iz godine u godinu nego raspored selidbe brezovog zviždka.

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1. INTRODUCTION

1.1 The study species

Willow Warbler (*Phylloscopus trochilus*) and Chiffchaff (*Phylloscopus collybita*) (Fig. 1) are two sympatric bird species which belong to order of the Leaf warblers (*Phylloscopus*), family of the Old World warblers (Sylviidae), sub-order songbirds (Oscines), order passerines (Passeriformes) (Clement et al. 2006). They are two most numerous and most widely distributed Leaf warbler species in the Western Palearctic (Cramp 1992), and both commonly breed across Europe (Fig. 2).

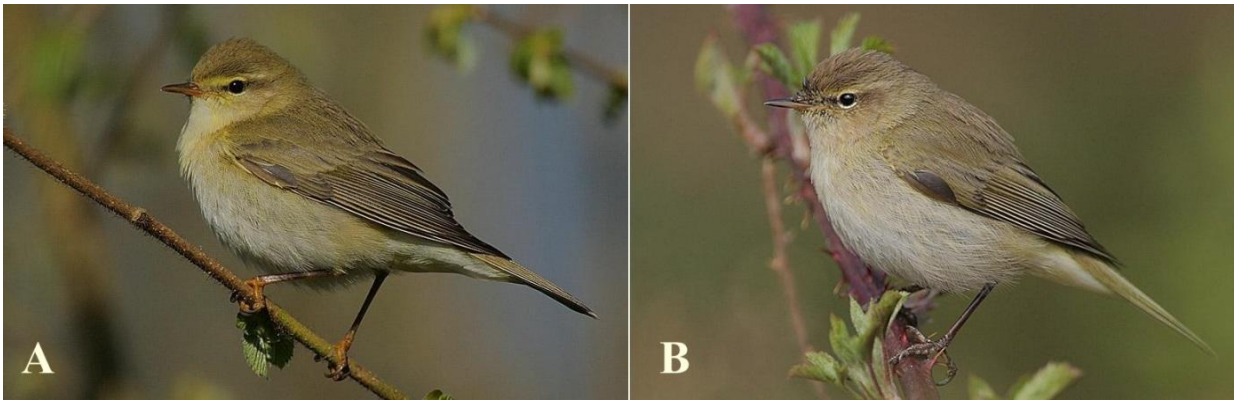

Figure 1. Study species. A: Willow Warbler (*Phylloscopus trochilus*), B: Chiffchaff (*Phylloscopus collybita*). (Photos: Steve Garvie, http://ibc.lynxeds.com/)

Willow Warbler is a polytypic species that includes three subspecies: *P. trochilus trochilus*, *P. t. acredula* and *P. t. yakutensis* (Svensson 1992). Subspecies *P. t. trochilus* and *P. t. acredula* are present in Poland during breeding and migration seasons. Breeding grounds of *P. t. trochilus* extend from western and central Europe to southern Scandinavia, while *P. t. acredula* breeds in central and eastern Europe, and in the northern part of Scandinavia and western Siberia (Ciach 2009). The populations of both subspecies of Willow Warbler that occur in Poland during spring (April-May) and autumn (September-November) migrations, and during breeding, are regular long-distance migrants (Bakken et al. 2003, Frasson and Hall-Karlsson 2008). Ringing of Willow Warblers in Europe has confirmed the existence of a migratory divide between two subspecies across central Scandinavia (Chamberlain et al. 2000, Bensch et al. 1999). The subspecies *P. t. trochilus*, breeding in southern Scandinavia, migrate along the western migration flyway to the wintering grounds in sub-Saharan west Africa, while northern-Scandinavian *P. t. acredula* migrate along the eastern migration flyway to winter in east, central and south-east Africa (Svensson 1992, Chamberlain et al.

2000, Bensch et al. 1999). Willow Warblers moult twice a year, both at the breeding and at the wintering grounds (Jenni and Winkler 2011).

Chiffchaff has a more complex intra-specific differentiation, with the status of some taxa still unresolved (Ciach 2009, Price 2010, Alström et al. 2013). The subspecies which breed in and migrate through Poland are *P. collybita collybita* and *P. c. abietinus*. Breeding grounds of *P. c. collybita* span from Iberian Peninsula to central and eastern Europe, and also reach southern Sweden (Hansson et al. 2000). European populations of *P. c. collybita* have a mixed migration system. Those breeding in the northern and central Europe are obligatory migrants, but those breeding more southerly are partial migrants, with a part of the population undertaking short-distance migration and a part being sedentary (Cramp 1992). Wintering grounds of *P. c. collybita* span western Europe, the Iberian Peninsula, the Mediterranean Sea basin, and west Africa (Bakken et al. 2003). *P. c. abietinus* breeds in northern and eastern Europe, from the northern part of Scandinavia to the Ural Mountains (Hansson et al. 2000). All European populations of this subspecies are regular long-distance migrants to the winter quarters in the eastern part of the Mediterranean Sea basin, Middle East and north-eastern and central Africa (Ciach 2009). Chiffchaffs migrate mainly during March-April and August-October, and undergo complete moult at their breeding grounds (Jenni and Winkler 2011, Bakken et al. 2003, Frasson and Hall-Karlsson 2008).

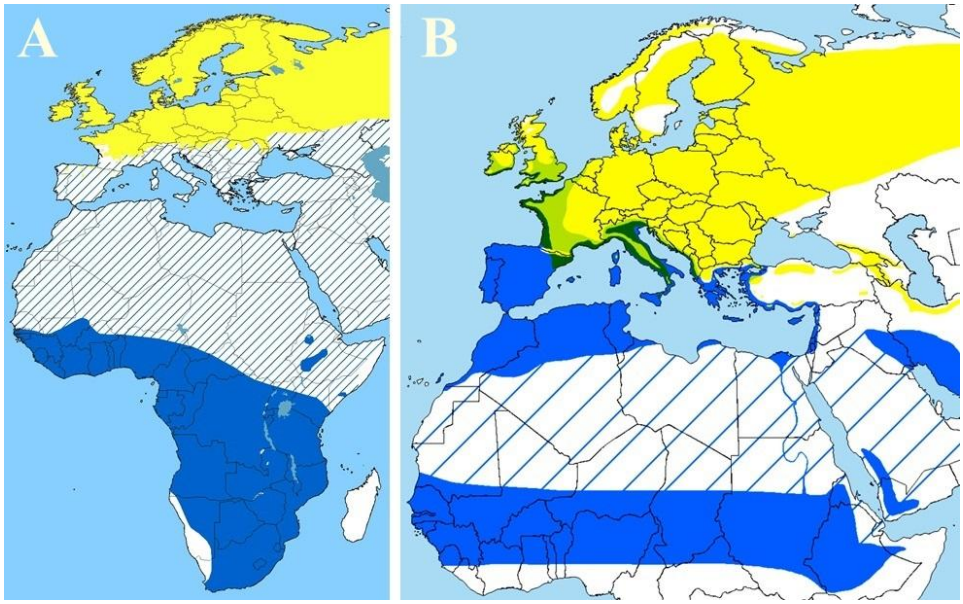

Figure 2. Distribution ranges of Willow Warbler (A) and Chiffchaff (B) in Europe and Africa. Yellow – breeding range, green – overlapping breeding and wintering range, blue – wintering range, striped – range where species are observed only on migration. (Adapted from: https://en.wikipedia.org/; accessed on 10 Jan 2016)

Willow Warbler and Chiffchaff are insectivorous species which can populate a wide range of habitats, and often coexist in the same areas, using the same feeding niches (Hanski & Tiainen 1991). Both species, however, have distinct habitat preferences. Willow Warbler prefers birch and willow thickets, tree plantations and early succession forest stages, shrubby areas of moor meadows and young forests. Chiffchaff is most commonly found in mixed and coniferous forests and riparian and alder swamp forests (Tiainen 1982, Hanski and Tiainen 1991, Svensson 1992). Their habitat preferences during the non-breeding period are not well known (Ciach 2009).

1.2 Sexing of birds based on sexual size dimorphism

Inter-specific and intra-specific differences in animals' morphology occur as a result of adaptation to environment and specific way of living. Differences in morphological traits within a single species are most frequently observed among geographically separate populations and between the sexes (James 1982). Sexual size dimorphism is common among birds, with males usually being larger than females, however a reverse sexual dimorphism also occurs in some bird orders. This size dimorphism often manifests in wing length, tarsus length or body mass (Svensson 1992). Most pronounced size dimorphism, with males 10-20% larger than females, is generally found among polygynous and lekking species (Payne 1984), while in socially monogamous species males are on average about 5% larger than females (Murphy 2007). While sexual selection is probably responsible for some differences in body size (Payne 1984, Székely et al. 2007), evolution of sexual size dimorphism might also be attributed to intra-specific competition for resources (Temeles et al. 2010), differential migration strategies (Leisler and Winkler 2003, Moore et al. 2003), parental roles and energetic strains during reproduction (Monaghan and Metcalfe 1986, Sandberg and Moore 1996), population density (Björklund and Lindén 1993) and other factors.

Aside from studies on its functional significance, numerous studies focus on size sexual dimorphism also to devise sexing criteria useful in other types of research (e.g. Ellrich et al. 2010, Huallacháin and Dunne 2010, Kulaszewicz et al. 2013, Henry et al. 2015). Sex determination of birds is crucial in behavioural (Balthazar and Ball 1995), ecological (Nyström 1990), conservation (Ito et al. 2003) and migration (Remisiewicz and Wennerberg 2006) studies. However, a majority of passerine species are monomorphic in plumage, which makes their sexing challenging (Price and Birch 1996). Most of the existing sexing methods

are limited to the season in which they can be applied (e.g. cloacal protuberance, brood patch or breeding behaviour), or are time-consuming, expensive or invasive (e.g. genetic analysis, hormone analysis, laparoscopy) (Svensson 1992, Griffiths et al. 1998, Eason et al. 2001, Risser 1971). Creating a simple sexing criteria for a species, based on morphology, is therefore advantageous. To develop and verify sexing criteria based on morphology, a sufficient sample of individuals of one species needs to be measured and sexed by molecular or another reliable sexing method (Griffiths et al. 1998, Morhina et al. 2013). Sexing criteria can then be derived from measurements of this sample of sexed birds, simply by dividing a bi-modal distribution or by application of logistical regression (Ellrich et al. 2010), discriminant analysis (Wojczulanis-Jakubas and Jakubas 2011), or principal component analysis (Remisiewicz and Wennerberg 2006).

In both Willow Warbler and Chiffchaff, sexual dimorphism is most pronounced in longer wings in males than in females (Tiainen 1982, Tiainen and Hanski 1985). Several authors suggested sexing criteria based on the wing length for Willow Warbler (Williamson 1967, Norman 1983, Tiainen & Hanski 1985, Svensson 1992, Ellrich et al. 2010) and for Chiffchaff (Ticehurst 1938, Williamson 1967, Lövei 1983, Tiainen & Hanski 1985, Geen 1988, Svensson 1992). However, only Ellrich et al. (2010) based their results on a reasonably large sample of DNA-sexed Willow Warblers, while similar study has not been conducted on Chiffchaffs. Furthermore, none of the previous studies were done on populations migrating through northern Poland. Ellrich et al. (2010) argue that the morphological sexing criteria generally cannot be applied over large geographical scale, because of geographical variation of subspecies and population, which makes the previously suggested sexing criteria for Willow Warblers and Chiffchaffs of limited application to these species migrating through northern Poland.

1.3. Timing of spring migration

Spring migration is generally considered more time constrained than autumn migration, because of numerous reproductive consequences that the timing of arrival at the breeding grounds may have for an individual (Smith and Moore 2004, Newton 2010). Birds fly faster in spring than in autumn and spend less time on stopover sites, which indicates a strong selection for saving time during spring migration (Drent et al. 2006, Nilsson et al. 2013, Alerstam 2011). Early arrival at the breeding grounds gives birds advantage in

competition for better quality territories (Kokko 1999). This allows early arriving individuals to start breeding earlier than those arriving late (Moore et al. 2005), and thus increase their reproductive performance (Smith and Moore 2004). Individuals of more favourable morphological traits are commonly observed to arrive and mate first, with the usual explanation stating that only birds in good condition can survive costs associated with early arrival (Kokko 1999, Møller et al. 2009).

Differential migration of the sexes is commonly observed in migratory birds (Coppack and Pulido 2009), and during spring migration usually occurs in the form of protandry (Morbey and Ydenberg 2001, Møller 2004, Rubolini et al. 2004, Kokko et al. 2006). Protandry refers to the earlier arrival at the breeding grounds of males than of females, caused by differences in their migration timing (Maggini and Barlein 2012). A number of hypotheses have been suggested to explain the evolutionary causes of protandry, however its adaptive significance is still not fully understood (Morbey and Ydenberg 2001). The two most supported, mutually inclusive explanations for evolution of protandry are the "rank advantage" hypothesis and the "mate opportunity" hypothesis (Morbey and Ydenberg 2001, Kokko et al. 2006, Møller et al. 2009). The rank advantage hypothesis, according to which competition for breeding sites amongst males drives selection for the early arrival, has received wide support, since in birds the territorial sex generally arrives earlier than the non-territorial sex (Nystörm 1997, Morbey and Ydenberg 2001, Tøttrup and Thorup 2008). The "mate opportunity" hypothesis relies on sexual selection, as early arrival improves males' chance to acquire a mate (Morbey and Ydenberg 2001, Kokko et al. 2006, Møller et al. 2009). Studies on the dependence of protandry on individuals' condition and the consequences of early arrival for fitness (Møller et al. 2009, Reudink et al. 2009), along with some theoretical models (Kokko et al. 2006), provide support for the "mate opportunity" hypothesis. However, Coppack and Pulido (2009) argue that available information on sexually differential migration is biased towards sexually dimorphic species, and point out the need for more studies on species monomorphic in plumage (such as Catry et al. 2004, Catry et al. 2005, Bowlin 2007, Edwards and Forbes 2007) to be included in comparative studies of protandry. Protandry is mostly achieved through spatial sexual segregation over latitude (Catry et al. 2007), with males wintering closer to the breeding grounds than females, and the differential onset of migration in the spring, with males starting their migration earlier than females (Maggini and Barlein 2012).

Differential migration in the form of protandry has been previously observed in both Willow Warbler and Chiffchaff (Nystörm 1997, Jakobsson 1988, Catry et al. 2005). Early arrival of males in these species may be advantageous for a number of reasons, for example it minimizes the risk of the previously owned territory being occupied by a new resident (Tiainen 1982), gives an opportunity to choose among a wider range of unoccupied territories (Nystörm 1997) and allows for the familiarization with the territory and its resources before breeding (Jakobsson 1988). Trends of protandry and sex-specific migration timing have not yet been studied for birds migrating through northern Poland.

Different weather conditions also have a great influence on the process of migration, and therefore on migration phenology (Berthold 2001, Elkins 2004, Newton 2010). According to Berthold (2001), migrants react directly to local weather conditions (wind, precipitation and temperature) rather than to global weather conditions as a whole. One of those local weather factors, temperature, directly influences the energy balance of birds during spring migration by affecting available food supplies through vegetation growth, insect activity and ice meltdown (Newton 2010). It is therefore advantageous for birds to adjust their migration timing to short-term and long-term variations in temperature (Elkins 2004, Marra et al. 2005, Newton 2010, Kölzsch et al. 2015). Short-distance migrants usually arrive at their breeding sites earlier, and are more responsive to the variation in weather conditions, than long-distance migrants (Berthold 2001, Bridge et al. 2010, Newton 2010). This pattern probably occurs because short-distance migrants winter closer to their breeding grounds than long-distance ones, and thus need less time to return there during spring migration, which allows them more flexibility in adjusting their passage to weather conditions (Kokko 1999, Newton 2010). Additionally, weather conditions steadily improve during spring, so the short-distance migrants are more likely to be delayed by poor weather than the long-distance migrants (Elkins 2004). Dorka (1966) observed year-to-year differences in arrival dates of Chiffchaffs in relation to weather conditions, but found no such differences in Willow Warblers. Several other studies described variation in median arrival dates of Willow Warblers in relation to climate change and weather conditions at their wintering grounds (Saino et al. 2007, Saino and Ambrosini 2008, Hedlund et al. 2015). Similar studies have not been conducted on populations of Chiffchaff and Willow Warbler migrating through northern Poland.

1.4 Study aims

This study focused on the analysis of populations of the Willow Warbler and the Chiffchaff that migrate through northern Poland in spring and autumn. The main aims of the study were:

1. To optimise the DNA-based sexing method for Willow Warbler and Chiffchaff.
2. To determine the extent of sexual dimorphism in these two species using DNA-based sexing method.
3. To develop the best morphological sexing criteria for the population mixtures of each species observed in northern Poland, using the measurements of the DNA-sexed individuals.
4. To determine sex differences in the migration timing of these two species, by applying the developed sexing criteria to the past ringing data.
5. To identify potential year-to-year differences in spring migration timing of males and females in both species, and to discuss them in the context of the effects of weather on migration.

2. STUDY AREA

Measurements and samples used in this study were collected at the Bukowo-Kopań ringing site, as a part of an ongoing research program "Operation Baltic", conducted since 1961 until present by the Bird Migration Research Station of the University of Gdańsk (Poland). The research area is situated along the southern Baltic Sea shore in northern Poland (Fig. 3). Data used in this study was collected during autumn and spring migration seasons in periods 2007-2009 at the ringing location Kopań and 2012-2015 at the location Bukowo. The exact locations of these two ringing sites are about 20 km apart along the same part of the coast, and thus are referred to as one ringing station Bukowo-Kopań. Data from 2010 (autumn) and 2011 (spring and autumn) was not included because Kopań did not operate long enough to cover full migration periods of both target species during those seasons.

Figure 3. Locations of the two ringing stations where the data was collected: Kopań (54º27'46"N, 16º24'38"E) and Bukowo (54º20'13"N, 16º14'36"E). (Adapted from: http://d-maps.com/; Google Earth, mapped on 29 Mar 2014, accessed on 18 Sep 2015)

The mist-nets catching birds at the Kopań ringing site were located in a mixed forest of birch (*Betula pendula*) and Scots pine (*Pinus sylvestris*) and over a wet meadow with scattered fruit bushes (*Crataegus* sp.*, Prunus avium, Prunus spinosa* and other). During the autumn migration seasons 2007-2009 additional mist netting location was used in reed beds (*Phragmites australis*) at the edge of Lake Kopań (Fig. 4). Willow Warblers and Chiffchaffs were mostly caught in the nets on a wet meadow.

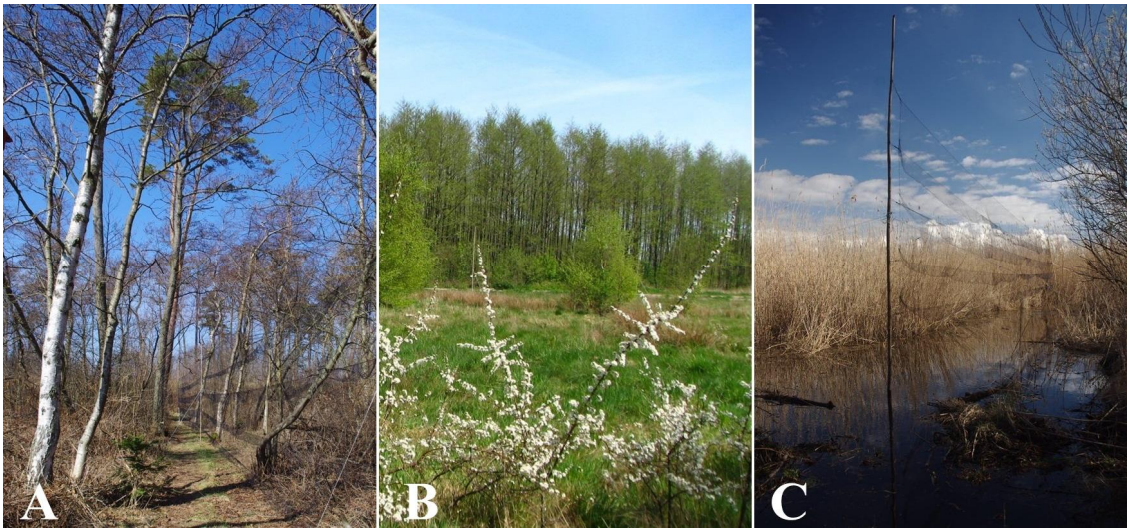

Figure 4. Habitats at mist net locations of Kopań ringing site. A: mixed forest of birch and Scots pine, B: wet meadow with scattered fruit bushes, C: reed beds. (Photos: Katarzyna Stępniewska)

Bukowo ringing site is located on a narrow spit between the Baltic Sea and Lake Bukowo, and covers a wide range of different habitats. Most of the nets were set up in a riparian forest of alder (*Alnus glutinosa*), ash (*Fraxinus excelsior*) and birch (*Betula pendula*), with undergrowth of fruit bushes (*Crataegus* sp., *Viburnum opulus, Prunus avium, Prunus spinosa, Sorbus acuparia* and other). Smaller number of nets were set up on yellow dunes and artificial sandbank overgrown by marram grass (*Ammophila arenaria*) and in a stripe of Scots pine trees (*Pinus sylvestris*) and scattered willow bushes (*Salix* sp.), as well as in the reed beds (*Phragmites australis*) at the bank of Lake Bukowo (Fig. 5).

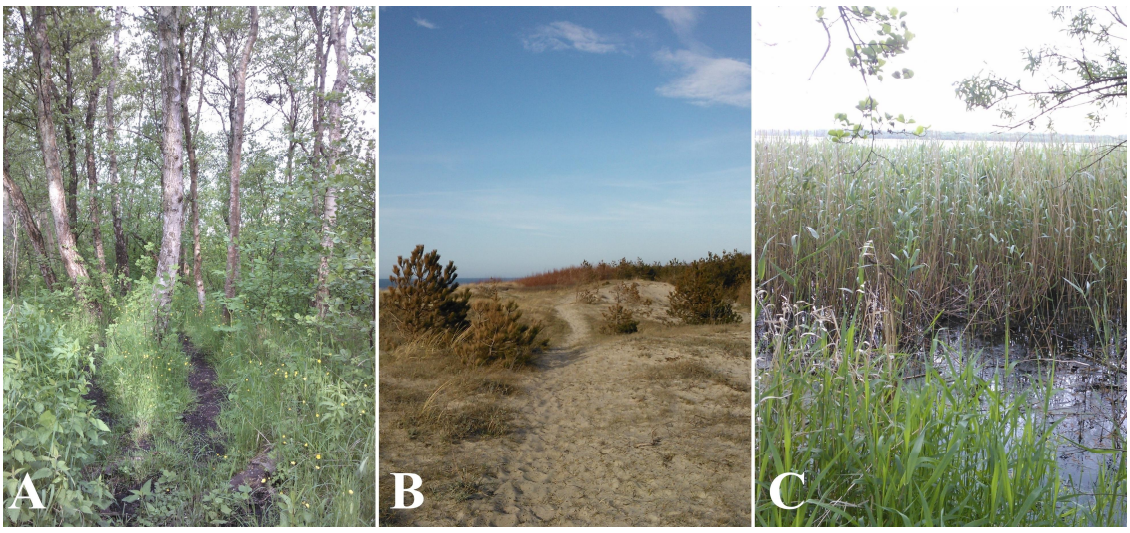

Figure 5. Habitats present at mist net locations of Bukowo ringing site. A: riparian forest with undergrowth of fruit bushes, B: yellow dunes with Scots pine and willow trees, C: reed beds. (Photos: Iva Šoštarić)

3. MATERIAL AND METHODS

3.1 Data and sample collection

Field work for this study was carried out at Bukowo-Kopań ringing site from 2007 to 2015. I collected the material in the field during the spring season of 2015. The two species in focus, Willow Warbler and Chiffchaff, were captured with standard ornithological mist-nets of 16 mm mesh during their spring (26 March – 26 May) and autumn (5 August – 5 October) migration through the study sites (Tab. 1).

Table 1. Operating schedule of Bukowo-Kopań ringing site during spring and autumn migration seasons analysed in this study.

Season	Location	Year	Start	End
spring	Bukowo	2015	27 Mar	26 May
	Bukowo	2014	21 Mar	27 May
	Bukowo	2013	25 Mar	22 May
	Bukowo	2012	26 Mar	21 May
	Kopań	2010	24 Mar	17 May
	Kopań	2009	25 Mar	17 May
autumn	Bukowo	2014	3 Aug	7 Nov
	Bukowo	2013	4 Aug	3 Nov
	Bukowo	2012	13 Aug	4 Nov
	Kopań	2009	13 Aug	9 Nov
	Kopań	2008	13 Aug	14 Nov
	Kopań	2007	12 Aug	14 Nov

Each captured individual was identified to the species, ringed and aged. Individuals were divided into three age categories: adult (A), immature (I) and unknown age (L). In autumn, adult Willow Warblers (birds in their second calendar year or older) can be identified by the white background colour of their underparts with occasional distinct yellow streaking on throat and breast, while immatures (birds in their first calendar year) usually have evenly coloured yellow or buff underparts. Adult Chiffchaffs are identified by fresh plumage with single-generation feathers and primary coverts with rounded tips and neat greenish edges, while the immatures have more worn flight feathers and a contrast between moulted and unmoulted greater coverts. Immature Willow Warblers can also be identified by the level of skull ossification until mid-September, and immatures of Chiffchaffs until the end of August (Svensson 1992, Jenni and Winkler 2011, Busse 2000). Both adult and immature Willow

Warblers undergo complete moult in their wintering quarters, therefore obtaining "adult" plumage before returning to their nesting site. For that reason during spring migration Willow Warblers in their third calendar year or older can no longer be distinguished from individuals in their second calendar year, and are therefore all regarded as adults. In spring, adult Chiffchaffs (birds in their third calendar year or older) can be recognized by well-preserved tips of primary feathers and broad and neat tips of primary coverts. The immature Chiffchaffs (birds in their second calendar year) may show abraded primary feathers, and occasionally moult limit in greater coverts and tail feathers. However, not all immatures can be identified, thus some birds are aged only as full-grown (Svensson 1992, Jenni and Winkler 2011).

The following set of measurements was taken for most of the captured individuals: wing length (according to Svensson's (1992) "maximum chord" method), distance between the longest primary and the first secondary feather ("Kipp's distance"; Kipp 1958), distance between the tips of the first and the second primary feathers, distance between the first primary and the longest greater covert, wing formula, tail length (Busse 2000), tarsus length (according to Svensson's (1992) "alternative method") and body mass. The tarsus was measured with calipers with the accuracy of 0.1 mm, and the rest of the measurements were taken with a ruler with the precision of 1 mm, the body mass was taken with an electronic balance with a precision of 0.1 g. In each individual the amount of fat in the furculum pit and on the belly was scored, according to the eight-score scale (Busse 2000). Most of these measurements were collected throughout the years on Bukowo-Kopań ringing station. But the tarsus was measured only during spring migration in 2015. Measuring was done by mutually calibrated bird ringers collaborating in the "Operation Baltic" research program. For some birds not all measurements were taken for various reasons (broken or very worn feathers, bird escaped during measuring etc.), so I chose only the individuals that had all the measurements required for my study available (Appendix, Tab. A1 and Tab. A2).

Blood and feather samples were collected only during the autumn migration in 2014 and spring migration in 2015. I collected the blood samples and measured the birds myself, after calibration with my supervisor Dr Magdalena Remisiewicz, during most of the spring season in 2015. Blood samples (5-20 µl) were taken from individual's brachial vein (Owen 2011) and preserved in 70 % ethanol in clearly marked vials. For three individuals samples of 6-7 feathers were taken from birds' flanks, and preserved in marked vials. Vials with both types of samples were stored in closed boxes, unexposed to light, and kept in a refrigerator at -4 °C for further analysis.

During the autumn migration in 2014, blood samples were collected from 82 Willow Warblers and 7 Chiffchaffs. In the following spring migration of 2015, blood samples were collected from 36 Willow Warblers and 51 Chiffchaff, and feather samples were collected from one Willow Warbler and two Chiffchaffs (Tab. 2).

Table 2. Total number of blood and feather samples collected from Willow Warblers and Chiffchaffs during spring (2015) and autumn (2014) migration season.

	Willow Warbler		**Chiffchaff**	
	spring 2015	autumn 2014	spring 2015	autumn 2014
adult	37	1	18	-
immature	-	66	29	7
unknown	-	15	6	-
total	**37**	**82**	**53**	**7**

During autumn 2014, samples were collected from most of the captured individuals in period from 6 August to 21 September, but there was no sampling during the rest of autumn migration of both species. During spring 2015, the sampling covered the whole migration of the target species, from 4 April to 26 May. Some individuals were not sampled for random reasons, such as poor condition of a bird or a short-term absence of the personnel trained in taking samples. Because these were random omissions of a few birds on random dates, we assumed that sampling covered the whole spring migration of both species and could be used for analysis of their migration timing.

3.2. Laboratory DNA sexing of birds from samples

The sex of the sampled individuals was determined using standardised PCR-based molecular techniques. These techniques rely on differences in intron sizes within genes to use them as markers for Z and W chromosomes. Amplicons produced from homogametic male genome (ZZ) are therefore distinguishable from those produced from heterogametic female genome (ZW), based on their size (Griffiths et al. 1998, Bantock et al. 2008). Most of the DNA samples were analysed using standard pair of primers for sexing of most birds, called P2/P8 (Griffiths et al. 1998). These primers are complimentary to intron regions of chromo-helicase-DNA gene (CHD) occurring on both sex chromosomes of most bird species. While the coding region of the gene is highly-conserved, its intron regions vary in length between the two sex chromosomes W and Z, enabling us to make a distinction between alleles

CHD-W (at chromosome W, shorter and exclusive to females) and CHD-Z (at chromosome Z, longer and present in both sexes). Primer pair P2/P8 produces amplicons of the size about 300-400 bp. For unknown reasons, 11 % of DNA samples could not be amplified using the primer pair P2/P8. After numerous adjustments were made to the existing protocol, it still failed to produce any results, so an alternative primer pair F2/R1 (Bantock et al. 2008) was applied. Primer pair F2/R1 binds to the intron region of the gene for avian mitochondrial ATP-synthase α-subunit (ATP5A1), and enables distinction of sexes based on the same basic principle as described for the P2/P8 primer pair. Amplicons produced with F2/R1 primers are 250-300 bp long.

3.2.1 Isolation of DNA

DNA was extracted from blood samples using the Blood Mini Kit (A&A Biotechnology). Firstly the blood clot was transferred from its initial vial into a labeled microcentrifuge tube, and 100 µL of Tris buffer (pH 8.5), 200 µL of universal lysis solution LT (containing chaotropic salts and non-ionic detergent) and 20 µL of Proteinase K were added. The entire mixture was incubated with gentle shaking in thermomixer for 20 min at 37 °C. The non-ionic detergent with Proteinase K facilitated lysis of the blood cells and release of DNA into the solution. After incubation the solution was transferred into previously labeled DNA purification columns, which were then centrifuged for 1 min at 13 000 rpm. In this step, chaotropic salts contained in universal lysis solution LT enabled DNA to bind to silica membrane of the purification column, allowing the contaminants (fragmented proteins, salts, polysaccharides, etc.) to wash through. Higher purity was insured by the two subsequent wash steps, during which a total of 900 µL of A1 washing solution was forced through the purification column at 13 000 rpm. The column was then transferred into a new, clearly labeled microcentrifuge tube, and 30 µL of low-salt Tris buffer was added directly onto the membrane in the column to elute DNA. The membrane was incubated for 5 min at room temperature, and centrifuged afterwards for 3 min at 13 000 rpm. The resulting solution of purified DNA was stored at 4 °C for further analysis.

From feather samples DNA was extracted from pulp cells inside the quill of feathers. Since this type of sample contains smaller amount of cells to isolate DNA from, I used Sherlock AX (A&A Biotechnology) DNA isolation kit for forensic and troublesome samples for this purpose. Firstly, quills of feathers were cut with sterile blades and transferred into a microcentrifuge tube containing 300 µL of deionised H_2O, 300 µL of L1.4 lysis buffer and 20 µL of Proteinase K. This mixture was then incubated in the thermomixer for 16 h, at 50 °C

and with gentle shaking. During the incubation DNA was released from the lysed cells. After incubation the sample was filtrated through filtration column for 1 min at 13 000 rpm, to separate quill leftovers from lysed cell material. The filtrate was then applied to Spin 10AX column, to bind the containing DNA onto column's ion-exchange membrane. This was followed by two wash steps, during which 600 µL of washing solution K2 was forced through the column at 8 000 rpm to remove various impurities (fragmented proteins, salts, polysaccharides, etc.) from the sample. DNA was then eluted from the membrane using 350 µL of high salt concentration elution buffer K3 and by centrifuging it at 8 000 rpm for 1 min. To precipitate the DNA, 5 µL of precipitation enhancer and 600 µL of isopropanol were added to the filtrate. After mixing, supernatant was removed and the remaining blue pellet containing DNA was washed with 70 % ethanol. The DNA pellet was then dried for 10 min and re-dissolved in 30 µL of Tris buffer (pH 8.5). Purified DNA solution was stored at 4 °C to await further analysis. Spectrophotometric analysis of concentration and purity of isolated DNA was performed on BioPhotometer (Eppendorf), using 2 µL of each DNA sample.

3.2.2 Polymerase chain reaction (PCR)

Purified DNA was afterwards amplified by PCR. The 16 µL reaction mix contained 2 µL of DNA sample, 1 µL of each primer (10 mM), 1 µL of additional $MgCl_2$ solution (25 mM), 3.5 µL of sterile-filtered H_2O and 7.5 µL of REDTaq® ReadyMix™ PCR Reaction Mix (Sigma-Aldrich). This PCR Reaction Mix served as a source of Taq DNA polymerase (0.06 unit/µL), reaction buffer (pH 8.3), dNTPs (0.4 mM) and $MgCl_2$ (3 mM). Although not strictly necessary, additional $MgCl_2$ was always included in the total reaction mix, as earlier tests showed that it significantly improves amplification success when used in the listed concentration. All PCRs were performed on T100™ Thermal Cycler (Bio-Rad); parameters used in reactions are listed in Table 3.

The sequences of the P2/P8 primers are as follows (Griffiths et al. 1998):

P2: 5'-TCTGCATCGCTAAATCCTTT-3'

P8: 5'-CTCCAAAGGATGAG[G/A]AA[T/C]TG-3'

Alternative, F2/R primer sequences are the following (Bantock et al. 2008):

F2: 5'-CCTCAGGACAAGGGAGGGGGAAATGTA-3'

R1: 5'-CCCCCTCCCTTGTCCTGAGGGGATTC-3'

Table 3. Cycling parameters for PCR reactions using P2/P8 and F2/R1 primer pairs.

	P2/P8			F2/R1		
step	**cycles**	**temp. [°C]**	**time [s]**	**cycles**	**temp. [°C]**	**time [s]**
initial denaturation	1	94	120	1	94	120
denaturation		94	30		94	45
annealing	40	50	30	29	48	45
extension		72	120		72	120
final extension	1	72	300	1	72	300

3.2.3 Electrophoresis of PCR products on agarose gel

The obtained PCR products were separated using horizontal gel electrophoresis. Amplicons originating from P2/P8 primer pair were separated on a 3.5 % agarose gel, while the shorter amplicons obtained from F2/R1 primers required denser, 4 % agarose gel (Basica LE, Prona). During the preparation of the gel, Midori Green Advanced DNA Stain (NIPPON Genetics) was added to enable subsequent DNA visualization. Midori Green is a dye which emits green fluorescence when bound to DNA, and is commonly used as a non-cancerogenic alternative to ethidium bromide. Gels were run in Bio-Rad electrophoresis chamber, conditions adjusted to the length of amplicons which were being separated (Tab. 4). After the electrophoresis, the gels were viewed under UV light (Fig. 6). The length of products was determined by comparison with the GeneRuler 50 bp DNA Ladder (Thermo Scientific).

Table 4. Conditions of electrophoresis used for amplicons obtained with the use of P2/P8 and F2/R1 primer pairs.

	P2/P8	**F2/R1**
region of amplicon lengths	300-400 bp	250-300 bp
concentration of agarose in gel	3.5 %	4 %
voltage	75 V	110 V
running time	120 min	60 min

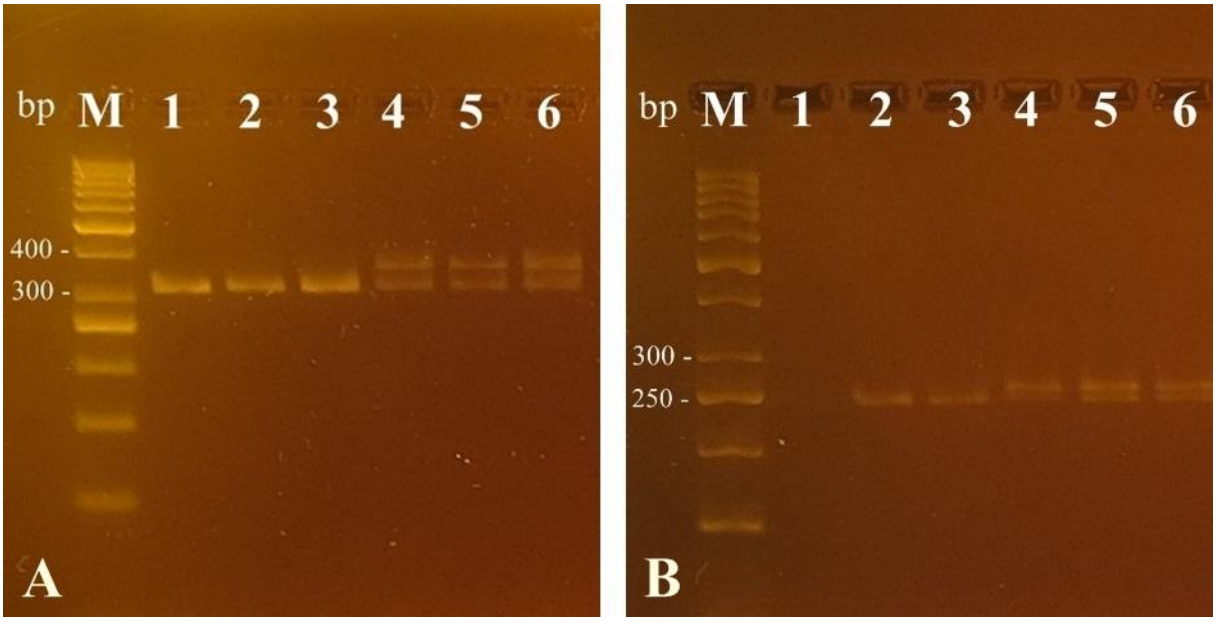

Figure 6. PCR products separated on agarose gel. A: amplicons obtained with P2/P8 primer pair (M: DNA ladder / molecular weight marker, 1-3: males, 4-6: females). B: amplicons obtained with F2/R1 primer pair (M: DNA ladder / molecular weight marker, 2-3: males, 4-6: females).

3.3. Analysis of morphological data

3.3.1. Initial comparison of chosen morphometric parameters

The measurements and the capture date of birds ringed in 2007-2010 and 2012-2015 were used for analysis of biometric features and migration timing of Willow Warblers and Chiffchaffs. The age classes (adults, immatures and birds of unknown age) were treated separately for the Willow Warbler, but were combined for the Chiffchaff, because of the low sample sizes of each age class.

Morphological measurements chosen for analysis were the following: wing length (W), distance between the first and the second primary feather (P_1P_2), distance between the longest primary feather and the first secondary feather (K), distance between the first primary and the longest greater covert (P_1GC) and tarsus length (S). Additionally, relative distance of first primary feather (P_{1r}) was calculated for some groups of individuals according to the formula: $P_{1r} = W - D_2 - P_1P_2$, where D_2 is distance from the tip of the second primary feather to the wing tip. To provide measure of sexual dimorphism, the Storer's dimorphism index (DI, Storer 1966) was calculated according to the formula: $DI = 100\ (f - m) / 0.5\ (f + m)$, where f and m are the mean values of analysed measurement in females and males, respectively.

Negative value of this index indicates that the males' measurement were greater than those of the females. Wing shape of individuals was described using three indices calculated from the wing formulas (Tab. 5): I_E – wing asymmetry index (Busse 1967), I_B – Busse's wing pointedness index (Busse 1967) and I_H – Hołyński's index (Hołyński 1965).

Table 5. Formulas and interpretations of wingtip shape indices used. W – wing length, ∑p – sum of primary distances proximal to wing tip, ∑d – sum of primary distances distal to wing tip.

Wingtip shape index	Formula	Original author's interpretation
Wing asymmetry index (I_E)	$I_E = 100\ \frac{\Sigma p - \Sigma d}{\Sigma p + \Sigma d}$	increasing with wing asymmetry
Busse's wing pointedness index (I_B)	$I_B = 100\ \frac{\Sigma p + \Sigma d}{W}$	increasing with wing pointedness
Hołyński's index (I_H)	$I_H = 100\ \frac{\Sigma p - \Sigma d}{W}$	increasing with wing pointedness and symmetry

Two-tailed t-test with equal variances, performed in Microsoft Excel 2010, was used to compare these measurements between age and sex groups of genetically sexed birds. Only the DNA-sexed individuals captured during the same migration season were compared. The accepted significance level was $P < 0.05$.

3.3.2 Deriving the sexing criteria

A single measurement that provided the best distinction between sexes (wing length) was used to establish the basic morphological sexing criteria for both species. To obtain a better resolution of sexing by combining the wing length with other measurements, I applied Principle Component Analysis (PCA, Zar 1999) using Statistica 12.0 software (StatSoft, Inc. 2013) and FactoMineR package (Husson et al. 2008) of R software ver. 3.1.3 (R Development Core Team 2007). For the PCA I combined wing length with those other measurements that showed highly significant difference between the sexes into a single parameter, the body size coefficient, derived as PC1, which could separate the sexes more successfully. The best PC1 was calculated using measurements of DNA-sexed birds, along with the available measurements of unsampled individuals from all of the seasons analysed. This way the PC1 values of DNA-sexed birds could be used to determine the morphological criteria to separate the sexes using the PC1 values calculated also for the larger sample of unsexed individuals. The different body size coefficients obtained by combinations of different measurements were

compared based on their efficiency to separate DNA-sexed individuals. The efficiency of sexing by a given PC1 was less than 100 % if the values of this coefficient overlapped between the DNA-sexed males and females, and so in this overlap zone they could not be reliably sexed based just on this PC1.

3.4 Analysis of migration timing

Timing of migration was compared between individuals that had been previously sexed morphologically or genetically. Capture dates of males and females ringed during all analysed migration seasons combined were compared by non-parametric Mann-Whitney U-test. Non-parametric Kruskall-Wallis test and post-hoc Mann-Whitney U-test were applied to compare migration dates of males and females among different years. These analyses were performed using Statistica 12.0 software (StatSoft, Inc. 2013), with the accepted significance level for all statistical analyses $P < 0.05$. I also compared changes in daily ringing numbers of Chiffchaffs with changes of average daily temperature during six spring migration seasons. The temperature was measured at the weather station in Łeba, located on the Baltic coast about 90 km from Bukowo-Kopań ringing site.

4. RESULTS

4.1 DNA sexing of Willow Warblers and Chiffchaffs

The concentration of isolated DNA averaged 35.5 ± 0.2 μg/mL for DNA from blood samples and 7.1 ± 0.4 μg/mL for DNA from feathers. Ratio of absorbance at 260 and 280 nm was used to assess purity of isolated DNA, and $A_{260/280}$ averaged 1.76 ± 0.35 (blood samples) and 1.82 ± 0.11 (feather samples). No significant differences in concentrations of isolated DNA between the two species were observed.

All of the 119 properly sampled individuals of Willow Warbler were sexed successfully (Tab. 7). Alternative primer pair F2/R1 was used in analysis of 19 samples which could not be amplified using the standard P2/P8 primer pair. Among 60 sampled individuals of Chiffchaff, 55 (92 %) were sexed successfully (Tab. 7). Most were amplified with P2/P8 primers, and 4 samples were amplified using the primer pair F2/R1. Five samples did not produce any results with either of the primers.

Table 7. Results of sexing of Willow Warblers and Chiffchaffs from blood and feather samples collected during autumn 2014 and spring 2015.

	Willow Warbler				**Chiffchaff**			
	spring 2015		autumn 2014		spring 2015		autumn 2014	
	male	female	male	female	male	female	male	female
adult	28	9	-	1	15	3	-	-
immature	-	-	37	29	11	12	3	3
unknown	-	-	10	5	7	1	-	-

4.2 Morphological sexing of Willow Warbler and Chiffchaff

4.2.1 Comparison of morphological features between the sexes

For the comparison of morphological features between sexes, three groups of birds with sufficient sample sizes of DNA-sexed individuals were chosen: immature Willow Warblers caught in autumn 2014, adult Willow Warblers caught in spring 2015, and Chiffchaffs (age groups combined) caught in spring 2015. To increase the sample sizes of the two latter groups I included into analyses of morphology one female Willow Warbler and one female Chiffchaff caught during incubation period and sexed by their prominent female-shaped brood patch (Svensson 1992).

4.2.1.1 Comparison of morphological features between the sexes in Willow Warbler

In adult Willow Warblers caught in spring 2015, males had on average 5.4 mm longer wing, 2.1 mm longer P_1P_2 distance and 0.7 mm longer tarsus when compared to the adult females. The differences between the sexes in the remaining morphological traits were not significant. In immature Willow Warblers caught in autumn 2014, males had on average 4.9 mm longer wing, 2.3 mm longer P_1P_2 distance and 1.7 mm longer K distance when compared to the immature females. There were no significant differences between immature males and females in the remaining morphological traits (Tab. 8).

Table 8. Comparison of morphological features of adult (spring 2015) and immature (autumn 2014) Willow Warblers between sexes. Mean, standard deviation (SD), sample size (N), and results of comparisons between the sexes by the t-test and the Storer's dimorphism index (DI), are provided. Measurements included are: W – wing length, P_1P_2 – distance between the first and the second primary feather, K – distance between the longest primary feather and the first secondary feather, P_1GC – distance between the first primary feather and the longest greater covert, and S – tarsus length; all expressed in millimetres. Indices compared are: I_E – wing asymmetry index, I_B – Busse's wing pointedness index and I_H – Hołyński's index. Statistically significant values are given in bold.

ADULTS

Measurement / Index	Males N = 28		Females N = 10		t-test		Storer's index
	mean	SD	mean	SD	t	P	DI
W	**69.8**	**1.3**	**64.4**	**1.3**	**11.41**	**1.63×10^{-13}**	**-8.04**
P_1P_2	**32.5**	**1.1**	**30.4**	**3.2**	**3.05**	**0.0042**	**-6.56**
K	17.1	1.2	16.6	1.1	1.06	0.2952	-2.80
P_1GC	3.8	1.1	4.4	1.4	-1.35	0.1845	14.07
S	**19.66**	**0.48**	**18.96**	**0.53**	**3.87**	**0.0004**	**-3.63**
I_E	69.95	4.77	68.49	5.26	0.80	0.4221	-2.12
I_B	52.05	4.45	53.62	4.07	-0.98	0.3349	0.33
I_H	36.49	4.63	36.84	4.99	-1.19	0.8446	0.94

IMMATURES

Measurement / Index	Males N = 37		Females N = 29		t-test		Storer's index
	mean	SD	mean	SD	t	P	DI
W	**68.6**	**1.2**	**63.7**	**1.9**	**12.71**	**3.66×10^{-19}**	**-7.38**
P_1P_2	**30.6**	**1.9**	**28.3**	**1.6**	**5.10**	**3.25×10^{-6}**	**-7.84**
K	**18.0**	**1.2**	**16.3**	**0.9**	**6.24**	**1.21×10^{-8}**	**-10.15**
P_1GC	4.6	1.7	4.5	1.2	0.30	0.765	-2.64
I_E	69.64	4.57	67.27	4.10	1.63	0.108	-2.59
I_B	50.32	4.70	50.42	4.56	-0.09	0.927	0.21
I_H	34.86	4.91	33.93	3.74	0.85	0.399	-2.72

I also compared the morphological features with each sex between the two age groups. The immature females had on average 1.2 mm shorter P_1P_2 distance than the adult females ($t = 2.29$, $P = 0.0275$). The differences between the age groups in females in the remaining morphological traits were not significant. The immature males compared to the adult males had on average 1.2 mm shorter wing ($t = 4.07$, $P = 0.0001$), 1.8 mm shorter P_1P_2 distance ($t = 4.48$, $P = 3.26\times10^{-5}$), 0.9 mm longer K measurement ($t = -3.05$, $P = 0.0033$) and 0.7 mm longer P_1GC distance ($t = -2.03$, $P = 0.0464$). Additionally, index I_B was on average 1.8 lower ($t = -2.13$, $P = 0.0365$) for immature males, indicating a more rounded wing shape, in comparison to adult individuals. Shorter P_1P_2 and longer P_1GC in immature males indicated a potential difference in length of first primary between the two age groups. An additional comparison of relative lengths of first primary feathers (P_{1r}) showed that immature males have 0.8 mm longer first primary when compared to adult birds ($t = -2.03$, $P = 0.0469$).

A single measurement that provided the best distinction between sexes of both age groups was the wing length. In immature Willow Warblers caught in autumn 2014, the females had wing length ≤ 66 mm, and the males ≥ 68 mm (Fig. 8). The overlap zone between the sexes spans wing lengths from 66 to 68 mm, and this includes 36 % (N = 24) of the sexed individuals from this season.

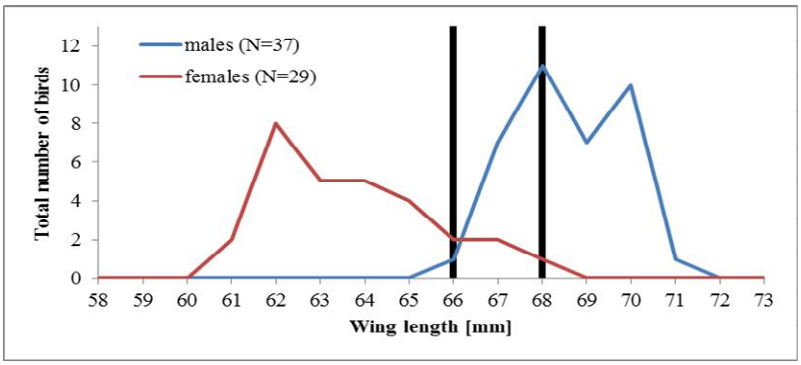

Figure 7. Distribution of wing lengths for immature Willow Warblers caught in autumn of 2014. Vertical lines indicate derived criteria for separating the sexes: females would be birds smaller than the left line value, males are birds larger than the right line value.

In adult Willow Warblers caught in spring 2015, females had wing length of ≤ 67 mm, while males had wing length of ≥ 68 mm, showing no overlap in size between the sexes (Fig. 8). Sexing criteria for both adult and immature Willow Warblers based on the wing length used in further analyses was derived from autumn 2014 data on immatures (Fig. 7), because of a small sample of adult females from spring 2015. Thus, hereafter adult and immature Willow Warbler with wing length ≤ 65 mm were regarded as females, the birds

with wing length ≥ 69 mm were regarded as males, and the individuals with wing lengths within the overlap zone of 66–68 mm remained unsexed.

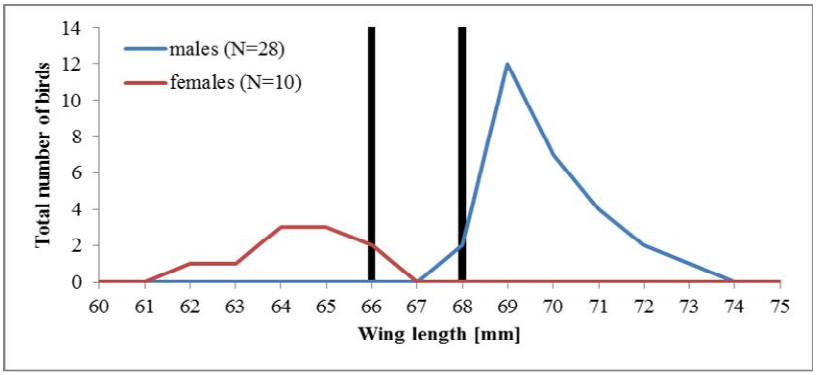

Figure 8. Distribution of wing lengths for adult Willow Warblers caught in spring of 2015. Vertical lines indicate derived criteria for separating the sexes (as in Fig. 7).

4.2.1.2 Comparison of morphological features between the sexes in Chiffchaff

In Chiffchaffs caught in spring 2015, the sexes differed significantly in almost all of the morphological traits compared. The male Chiffchaffs compared to females had on average 5.7 mm longer wing, 2.3 mm longer P_1P_2 distance, 1.1 mm longer K distance, 1.0 mm longer P_1GC distance and 1.5 mm longer tarsus. Additionally, males had on average 11.3 higher I_E index and 4.3 higher I_H index, in comparison to females (Tab. 9).

Table 9. Comparison of morphological features of Chiffchaffs from spring 2015 between sexes. Mean, standard deviation (SD), sample size (N), results of comparisons between the sexes by the t-test, and the Storer's dimorphism index (DI), are provided. Measurements included are: W – wing length, P_1P_2 – distance between the first and the second primary feather, K – distance between the longest primary feather and the first secondary feather, P_1GC – distance between the first primary feather and the longest greater covert and S – tarsus length; all expressed in millimetres. Indices compared are: I_E – wing asymmetry index, I_B – Busse's wing pointedness index and I_H – Hołyński's index. Statistically significant values are given in bold

Measurement / Index	Males			Females			t-test		Storer's index
	mean	SD	N	mean	SD	N	t	P	DI
W	**62.3**	**1.9**	**33**	**56.6**	**1.3**	**17**	**11.22**	**5.11×10^{-15}**	**-9.51**
P_1P_2	**25.2**	**1.2**	**31**	**23.0**	**1.3**	**15**	**5.78**	**7.2×10^{-7}**	**-9.36**
K	**12.0**	**1.3**	**29**	**10.9**	**1.0**	**11**	**2.47**	**0.0179**	**-9.52**
P_1GC	**6.2**	**1.1**	**30**	**5.1**	**1.0**	**15**	**3.03**	**0.0041**	**-18.29**
S	**19.77**	**0.63**	**33**	**18.27**	**0.53**	**15**	**8.05**	**2.51×10^{-10}**	**-7.87**
I_E	**37.83**	**9.93**	**27**	**26.54**	**12.59**	**12**	**3.01**	**0.0046**	**-35.05**
I_B	36.06	4.75	27	35.05	5.92	12	0.57	0.5722	-2.85
I_H	**13.74**	**4.06**	**27**	**9.43**	**5.07**	**12**	**3.08**	**0.0073**	**-37.25**

A single measurement that provided the best distinction between the sexes was the wing length. In Chiffchaffs from spring 2015, the females had wing length ≤ 59 mm, the males had wing length of ≥ 59 mm, the overlap zone of the wing length 59 mm included 4 % of sexed birds (N = 2) (Fig. 9). The sexing criteria for Chiffchaffs based on the wing length used in further analyses were derived from this data, but extending the zone of overlap, where birds cannot be sexed with certainty, for one millimetre either side. Thus, in further analysis Chiffchaffs of all age groups with wing length of ≤ 57 mm were regarded as females, birds with wing length of ≥ 61 mm were regarded as males, and the individuals with wing lengths of 58-60 mm remained unsexed.

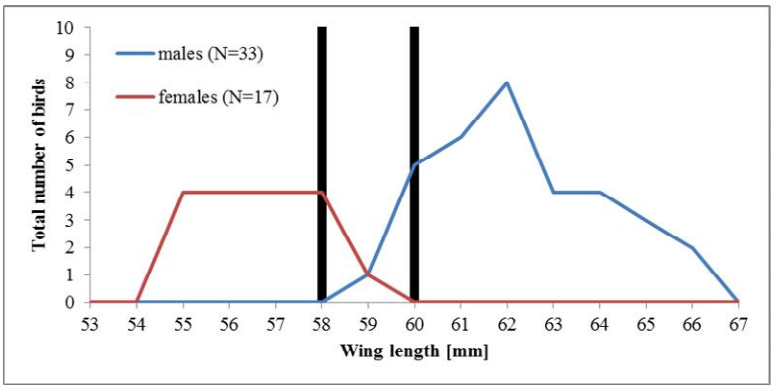

Figure 9. Distribution of wing lengths for Chiffchaffs caught in spring of 2015. Vertical lines indicate derived criteria for separating the sexes (as in Fig. 7).

4.2.2 Sexing criteria based on body size coefficients (PC1)

Among single measurements the wing length provided the best, but not 100 % efficient, distinction between the sexes in both species. So the next step was to attempt a better resolution of sexing by combining the wing length with other measurements, using the Principal Component Analysis (PCA) to derive body size coefficients (PC1). The PC1 values of DNA-sexed birds were used to determine the criteria to separate the sexes using the PC1 values calculated also for the larger sample of unsexed individuals.

4.2.2.1 Sexing criteria based on body size coefficients (PC1) for Willow Warbler

For adult Willow Warblers captured in spring 2015, body size coefficients (PC1) were calculated using different combinations of W, P_1P_2 and S measurements. The PCA sample included DNA-sexed birds from spring 2015, along with the unsampled individuals from all six spring seasons covered in this study (2009, 2010, 2012, 2013, 2014, 2015) (Tab. 10).

Table 10. Comparison of efficiency of different morphological parameters for sexing of Willow Warblers captured during spring of 2015. The combination of measurements used to calculate each option of PC1 index is given in the brackets. Sample size of DNA-sexed males (m), DNA-sexed females (f), and unsampled birds (u) used in the PCA is provided.

sexing parameter	criteria to determine females	criteria to determine males	% of individuals correctly sexed with these criteria	PCA sample size		
				m	f	u
W	W ≤ 65 mm	W ≥ 69 mm	90 %	28	10	-
PC1 (W+P_1P_2)	PC1 < -0.4	PC1 > -0.2	100 %	28	10	355
PC1 (W+P_1P_2+S)	PC1 < -0.6	PC1 > -0.4	100 %	28	10	7
PC1 (W+S)	PC1 < -0.6	PC1 > -0.4	92 %	28	10	7

The most efficient body size coefficient (PC1), the one that combined W and P_1P_2 (Tab. 10), was calculated according to the following equation: PC1 = (0.707×W) + (0.707×P_1P_2), coefficient of determination of the equation was $R^2 = 0.85$. Loadings for the variables were: W = 0.50, P_1P_2 = 0.50. I chose the PC1 (W+P_1P_2) as the best coefficient because of fewer measurements involved. The gap in the values, which separated the sexes was -0.4 < PC1 (W+P_1P_2) < -0.2. Based on that, the Willow Warblers with PC1 (W+P_1P_2) < -0.4 would be females, and those with PC1 (W+P_1P_2) > -0.2 would be males (Fig. 10).

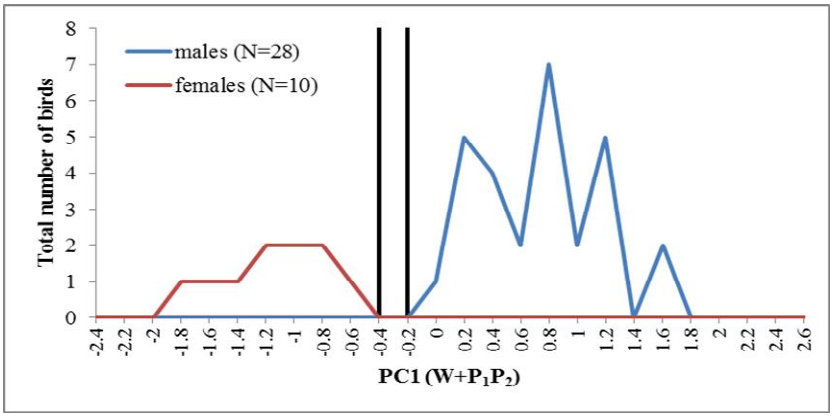

Figure 10. Distribution of PC1 (W+P_1P_2) values for DNA-sexed Willow Warblers captured during spring of 2015. Vertical lines indicate derived criteria for separating the sexes (as in Fig. 7)

The second best coefficient PC1 (W+P_1P_2+S) was calculated according to the following equation: PC1 = (0.61×W) + (0.60×P_1P_2) + (0.52×S), its coefficient of determination was $R^2 = 0.72$. Loadings for the two variables were: W = 0.37, P_1P_2 = 0.36 and S = 0.26. Based on the distribution of this PC1 (W+P_1P_2+S) of DNA-sexed individuals, the gap in values that separated the sexes was -0.6 < PC1 < -0.4. Therefore the birds with values of PC1 (W+P_1P_2+S) < -0.6 would be females, the birds with PC1 (W+P_1P_2+S) > -0.4 would

be males (Fig. 11). However, the body size coefficient PC1 (W+P_1P_2+S) could not be applied to sex adult Willow Warblers from the past data, because S measurement was not taken during the previous seasons. Therefore, for further analyses of adult Willow Warblers I only considered the two sexing criteria described earlier, 1) based on the wing length, and 2) based on PC1 (W+ P_1P_2).

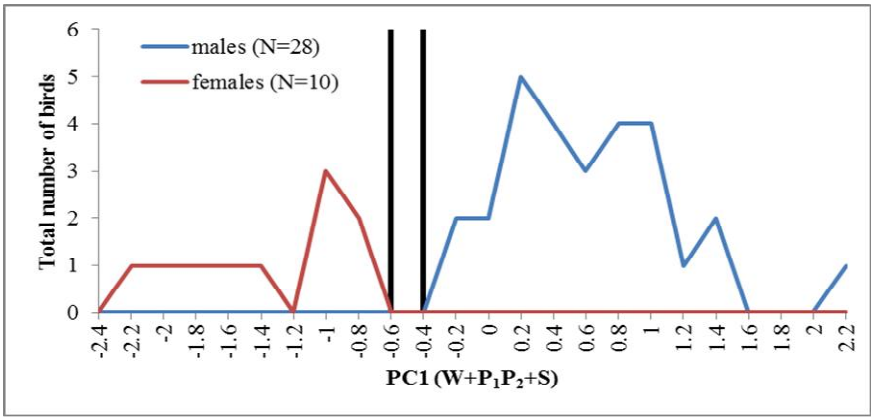

Figure 11. Distribution of PC1 (W+P_1P_2+S) values for DNA-sexed Willow Warblers captured during spring of 2015. Vertical lines indicate derived criteria for separating the sexes (as in Fig. 7).

In immature Willow Warblers captured in autumn 2014, the body size coefficients (PC1) were calculated using W, P_1P_2 and K measurements. The PCA sample included DNA-sexed birds from autumn 2014, along with the unsampled individuals from all six autumn seasons covered in this study (2007, 2008, 2009, 2012, 2013, 2014) (Tab. 11).

Table 11. Comparison of efficiency of different morphological parameters for sexing of immature Willow Warblers captured during autumn migration season of 2014. The combination of measurements used to calculate each option of PC1 index is given in the brackets. Sample size of DNA-sexed males (m), DNA-sexed females (f), and unsampled birds (u) used in the PCA is provided.

sexing parameter	criteria to determine females	criteria to determine males	% of individuals correctly sexed with these criteria	PCA sample size		
				m	f	u
W	W ≤ 65 mm	W ≥ 69 mm	64 %	37	29	-
PC1 (W+P_1P_2)	PC1 < -0.4	PC1 > 0.4	68 %	36	29	875
PC1 (W+K)	PC1 < -0.2	PC1 > 0.4	68 %	37	29	360
PC1 (W+P_1P_2+K)	PC1 < -0.4	PC1 > 0.4	65 %	36	29	358

The best coefficient PC1 (W+P_1P_2) was calculated according to the following equation: PC1 = (0.707×W) + (0.707×P_1P_2), its coefficient of determination was R^2 = 0.85. Loadings for the two variables were: W = 0.50 and P_1P_2 = 0.50. Based on the distribution of this PC1

($W+P_1P_2$) of DNA-sexed individuals, the range of values separating the sexes was -0.4 < PC1 < 0.4, and therefore the birds with values of PC1 ($W+P_1P_2$) < -0.4 would be females, the birds with PC1 ($W+P_1P_2+S$) > 0.4 would be males (Fig. 12).

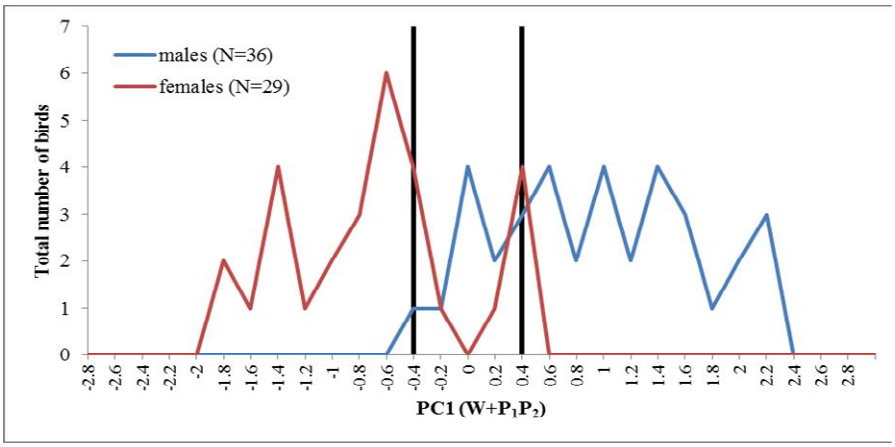

Figure 12. Distribution of PC1 ($W+P_1P_2$) values for DNA-sexed immature Willow Warblers captured during autumn of 2014. Vertical lines indicate derived criteria for separating the sexes (as in Fig. 7).

The second best body size coefficient for sexing immature Willow Warblers from autumn 2014 was PC1 that combined W and K measurements. This PC1 was calculated according to the following equation: PC1 = (0.707×W) + (0.707×K), coefficient of determination of the equation was $R^2 = 0.62$. The loadings for the two variables were: W = 0.50, K = 0.50. Based on the distribution of this best PC1 (W+K) of DNA-sexed individuals, the range of the PC1 values sexes overlap was -0.2 < PC1 < 0.4, and therefore birds with PC1 < -0.2 would be females, and the birds with PC1 > 0.4 would be males (Fig. 13).

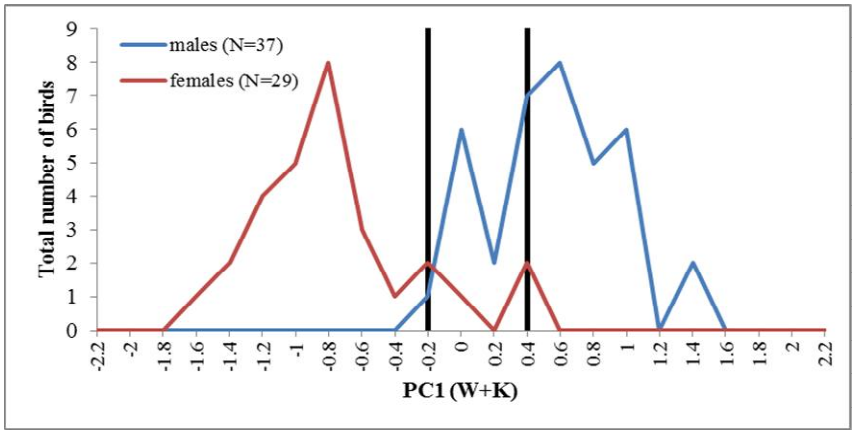

Figure 13. Distribution of PC1 (W+K) values for DNA-sexed immature Willow Warblers captured during autumn of 2014. Vertical lines indicate derived criteria for separating sexes (as in Fig. 7).

4.2.2.2 Sexing criteria based on body size coefficients (PC1) for Chiffchaff

For analysis of Chiffchaffs captured during spring 2015, body size coefficients (PC1) were calculated using W, P_1P_2 and S measurements. The sample for PCA included

DNA-sexed birds from spring 2015, along with the unsampled individuals from all six spring seasons covered in this study (2009, 2010, 2012, 2013, 2014, 2015) (Tab. 12).

Table 12. Comparison of efficiency of different combinations of morphological parameters for sexing of Chiffchaffs captured during spring migration season of 2015. The combination of measurements used to calculate each option of PC1 index is given in the brackets. Sample size of DNA-sexed males (m), DNA-sexed females (f), and unsampled birds (u) used in the PCA is provided.

sexing parameter	criteria to determine females	criteria to determine males	% of individuals correctly sexed with these criteria	PCA sample size		
				m	f	u
W	W ≤ 57 mm	W ≥ 61 mm	78 %	33	17	-
PC1 (W+S)	PC1 < -0.6	PC1 > -0.2	96 %	33	15	17
PC1 (W+P_1P_2+S)	PC1 > 0.4	PC1 < 0.2	87 %	31	14	16
PC1 (W+P_1P_2)	PC1 < -0.6	PC1 > 0.2	78 %	31	15	387

The most efficient body size coefficient for sexing Chiffchaffs was PC1 that combined W and S measurements. It was calculated according to the following equation: PC1 = (0.707×W) + (0.707×S), its coefficient of determination was $R^2 = 0.87$. The loadings for the two variables were: W = 0.50, S = 0.50. Based on the distribution of this best PC1 (W+S) of DNA-sexed individuals, the PC1 values of the sexes overlapped in the range -0.6 < PC1 < -0.2 (Fig. 14).

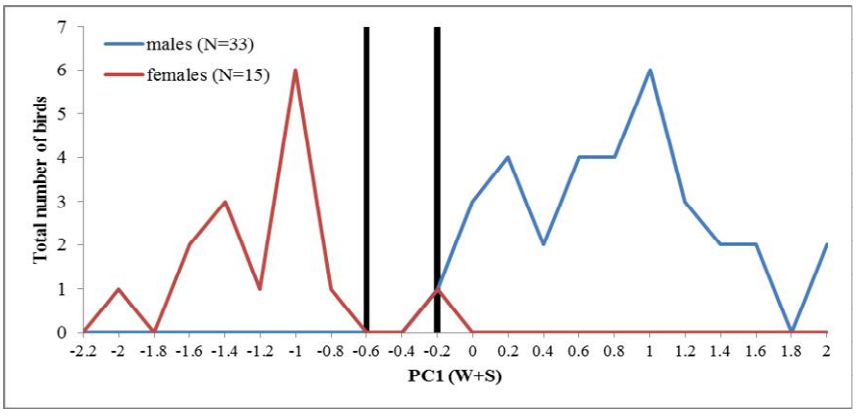

Figure 14. Distribution of PC1 (W+S) values for DNA-sexed Chiffchaffs captured during spring of 2015. Vertical lines indicate derived criteria for separating the sexes (as in Fig. 7).

However, this body size coefficient PC1 (W+S) could not be applied to sex Chiffchaffs from the past data, because S measurement was not taken during the previous seasons. Therefore, in further analyses I used the sexing criteria based on the wing length and based on PC1 (W+ P_1P_2), described previously.

4.2.3 Efficiency of sexing birds caught in past spring seasons using proposed criteria

4.2.3.1 Efficiency of sexing Willow Warblers using proposed criteria

The most efficient sexing parameter for both adult Willow Warblers caught in spring 2015 and immature Willow Warblers caught in autumn 2014, was PC1 combining W and P_1P_2 measurements (Tab. 10 and 11). This best PC1 was then calculated for 355 unsampled Willow Warblers captured during six spring migration seasons (Fig. 15). Based on obtained PC1 ($W+P_1P_2$) values and the criteria for adult birds captured during spring season (proposed in the previous paragraph), I was able to sex 91 % of Willow Warblers.

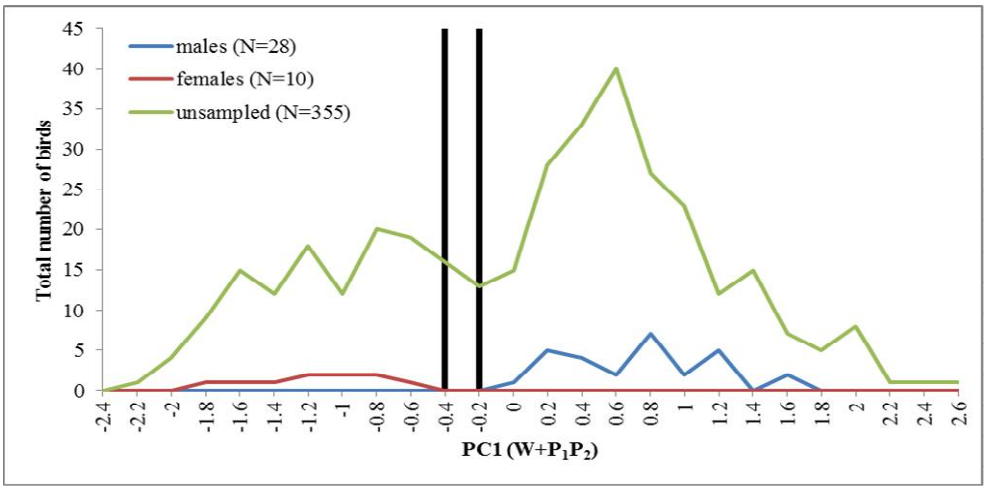

Figure 15. Distribution of PC1 ($W+P_1P_2$) values for Willow Warblers captured during six spring migration seasons (2009, 2010, 2012, 2013, 2014, 2015). Blue line – DNA-sexed males captured during spring 2015; red line – DNA-sexed females captured during spring 2015; green line – unsampled individuals captured during all six spring seasons, combined. Vertical lines indicate derived criteria for separating the sexes: females would be birds smaller than the left line value, males are birds larger than the right line value.

W and P_1P_2 measurements were also combined for 1108 unsampled Willow Warblers captured during six autumn migration seasons (Fig. 16). Using the PC1 factor obtained for each individual with the previously proposed criteria for sexing immatures caught in autumn, I was able to sex 66 % of Willow Warblers.

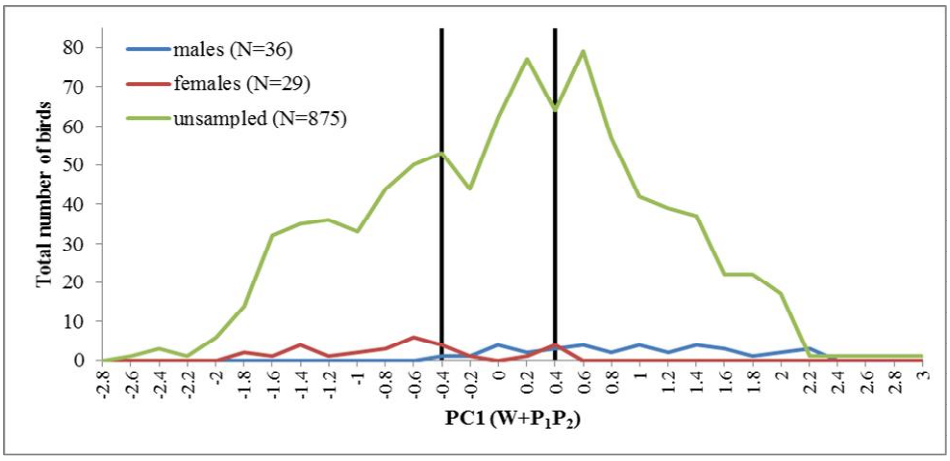

Figure 16. Distribution of PC1 ($W+P_1P_2$) values for Willow Warblers captured during six autumn migration seasons (2007, 2008, 2009, 2012, 2013, 2014). Blue line – DNA-sexed males captured during spring 2015; red line – DNA-sexed females captured during spring 2015; green line – unsampled individuals captured during all six autumn migration seasons, combined. Vertical lines indicate derived criteria for separating the sexes (as in Fig. 15).

The criteria based only on wing length described earlier were also applied for comparison to sex Willow Warblers captured during six spring (Fig. 17) and autumn (Fig. 18) migration seasons. Willow Warblers captured during spring seasons were sexed with 80 % efficiency, and birds captured during autumn seasons were sexed with 62 % efficiency.

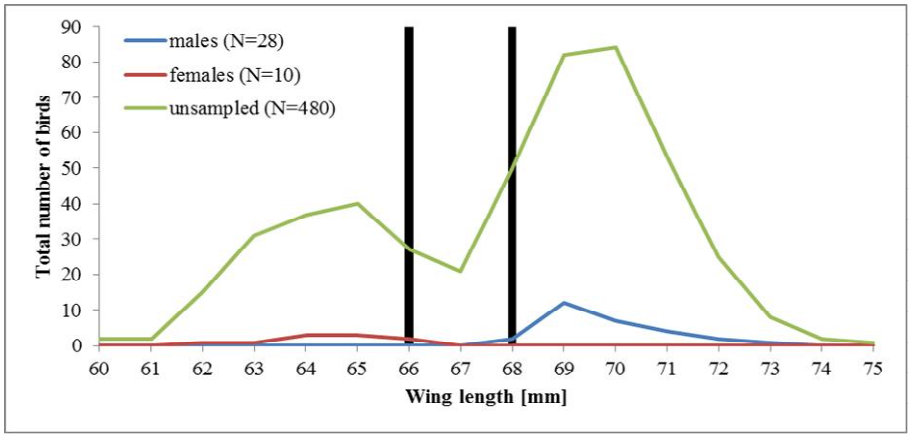

Figure 17. Distribution of wing lengths for adult Willow Warblers captured during six spring migration seasons (2009, 2010, 2012, 2013, 2014, 2015). Blue line – DNA-sexed males captured during spring 2015; red line – DNA-sexed females captured during spring 2015; green line – unsampled individuals captured during all six spring seasons, combined. Vertical lines indicate derived criteria for separating the sexes (as in Fig. 15).

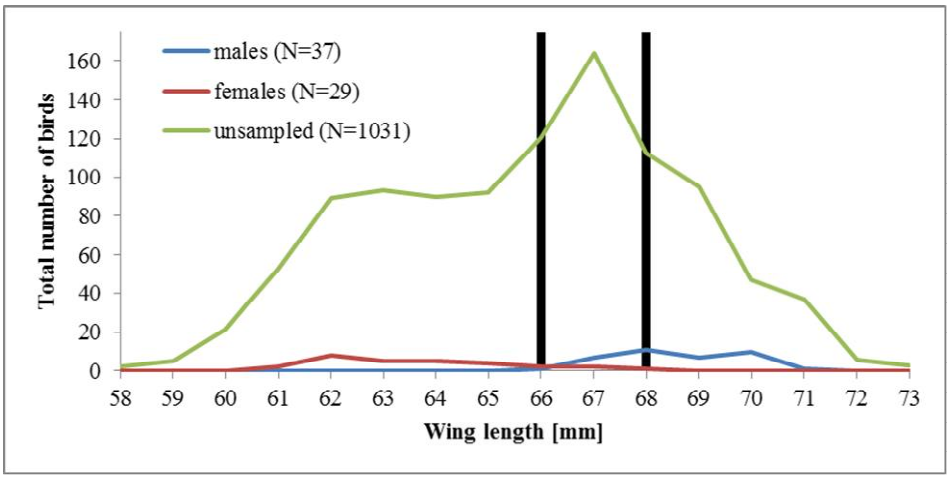

Figure 18. Distribution of wing lengths for immature Willow Warblers captured during six autumn migration seasons (2007, 2008, 2009, 2012, 2013, 2014). Blue line – DNA-sexed males captured during spring 2015; red line – DNA-sexed females captured during spring 2015; green line – individuals of undetermined sex captured during all six autumn migration seasons, combined. Vertical lines indicate derived criteria for separating the sexes (as in Fig. 15).

PC1 combining W and P_1P_2 measurements was the most efficient parameter for sexing of adult and immature Willow Warblers captured during past migration seasons analysed. However, the sample size of birds with both W and P_1P_2 measurement was considerably smaller compared to the sample of birds with W measurement alone. Therefore I was able to sex more individuals with the use of wing length based criteria (Tab. 13). Combining the use of both these criteria, I was able to sex 651 unsampled immature and 436 unsampled adults Willow Warblers, which I used later to analyse the migration timing of the sexes.

Table 13. Efficiencies of proposed sexing parameters in sexing of previously DNA-sexed and unsampled adult and immature individuals of Willow Warbler from six spring (2009, 2010, 2012, 2013, 2014, 2015) and six autumn migration seasons (2007, 2008, 2009, 2012, 2013, 2014). Symbols as in Tab. 10.

	sexing parameter	efficiency in sexing DNA-sexed birds	efficiency in sexing unsampled birds	total number of birds sexed
Adults	PC1 (W+P_1P_2)	100 %	91 %	326
	W	90 %	80 %	382
	PC1 (W+P_1P_2) and W	100 %	90 %	475
Immatures	PC1 (W+P_1P_2)	68 %	66 %	576
	W	64 %	61 %	633
	PC1 (W+P_1P_2) and W	71 %	76 %	790

4.2.3.2 Efficiency of sexing Chiffchaffs using proposed criteria

Wing length and PC1 that combined W and P_1P_2 measurements, described earlier, were the two most efficient parameters for sexing Chiffchaffs caught in spring 2015 (Tab. 12). PC1 ($W+P_1P_2$) was calculated for 384 unsampled Chiffchaffs captured during six spring migration seasons (Fig. 19). Based on the criteria proposed in the previous paragraph I was able to sex 85 % of unsampled Chiffchaffs according to their PC1 ($W+P_1P_2$) values.

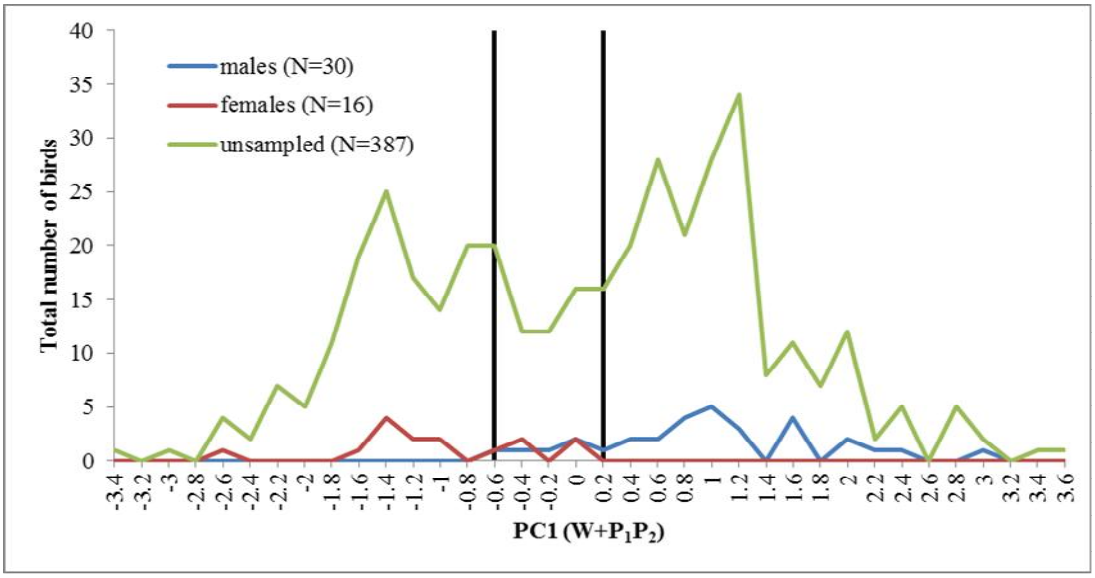

Figure 19. Distribution of PC1 ($W+P_1P_2$) values for Chiffchaffs captured during six spring migration seasons (2009, 2010, 2012, 2013, 2014, 2015). Blue line – DNA-sexed males captured during spring 2015; red line – DNA-sexed females captured during spring 2015; green line – unsampled individuals captured during all six autumn migration seasons, combined. Vertical lines indicate derived criteria for separating the sexes (as in Fig. 15).

The criteria based a single measurement (the wing length) were the simpler option which could be applied to a larger sample of birds (N=515). With the use of wing length based criteria I was able to sex 78 % of Chiffchaffs captured during six spring migration seasons (Fig. 20).

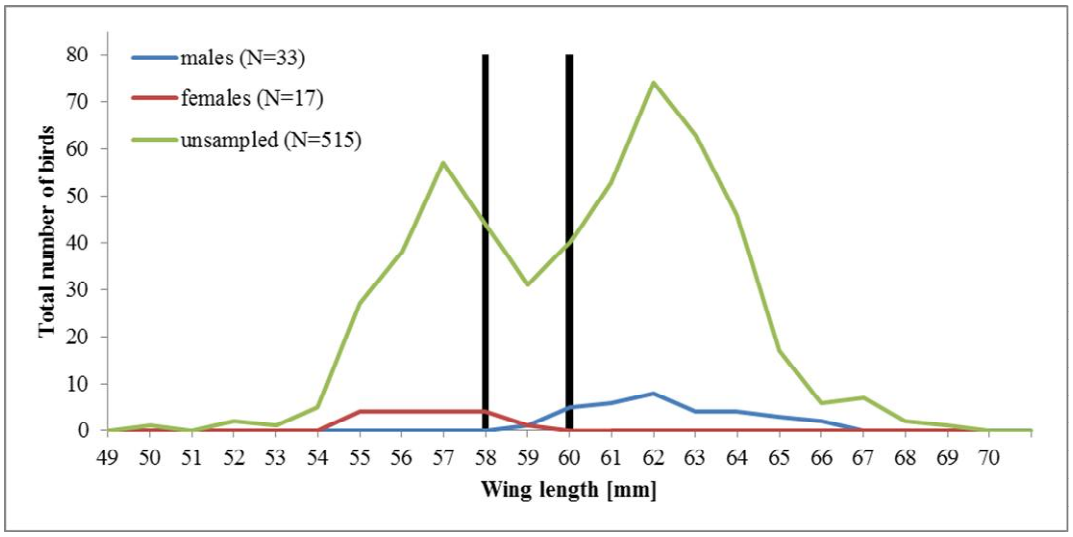

Figure 20. Distribution of wing lengths for Chiffchaffs captured during six spring migration seasons (2009, 2010, 2012, 2013, 2014, 2015). Blue lines – DNA-sexed males captured during spring 2015; red lines – DNA-sexed females captured during spring 2015; green lines – individuals of undetermined sex captured during all six spring seasons, combined. Vertical lines indicate derived criteria for separating the sexes (as in Fig. 15).

Both parameters were equally adequate while I tested their sexing efficiency on the sample of the previously DNA-sexed birds, which allowed me to verify sexing according to the proposed criteria based on measurements against sexing by DNA. But PC1 ($W+P_1P_2$) was more efficient than wing length (W) in overall sexing the unsampled individuals (Tab. 14). However, bigger sample size of birds with only the wing measurement (but not P_1P_2) allowed me to sex more individuals using the simpler method based on the wing length. Combining the use of both these criteria, I was able to sex 481 unsampled Chiffchaffs, which I used later to analyse the migration timing of the sexes.

Table 14. Efficiencies of proposed parameters in sexing of previously DNA-sexed and unsampled Chiffchaffs from six spring migration seasons (2009, 2010, 2012, 2013, 2014, 2015). Symbols as in Tab. 10.

sexing parameter	efficiency in sexing DNA-sexed birds	efficiency in sexing unsampled birds	total number of birds sexed
W	78 %	78 %	400
PC1 ($W+P_1P_2$)	78 %	85 %	327
PC1 ($W+P_1P_2$) and W	78 %	88 %	481

4.3 Spring migration timing of males and females

4.3.1 Spring migration timing of Willow Warbler

The sexing criteria developed and evaluated in the previous chapters were used to analyse the timing of spring migration for 475 morphologically and genetically sexed adult Willow Warblers, which were 92 % of all individuals captured and measured during six spring migration seasons (Appendix p. I, Tab. A1). In all six spring seasons combined the earliest migrants were the males, which were arriving from the 6 April, while the earliest females occurred 5 days later (Figs. 21, 22). The second highest peak of male's migration occurred on 19 April, while the second highest peak of females' migration occurred on 23 April. Males had the main peak of migration on 27 April, and the main, second, peak of females' migration occurred on 12 May. The latest migrants (both males and females) were recorded after 24 May (Fig. 21).

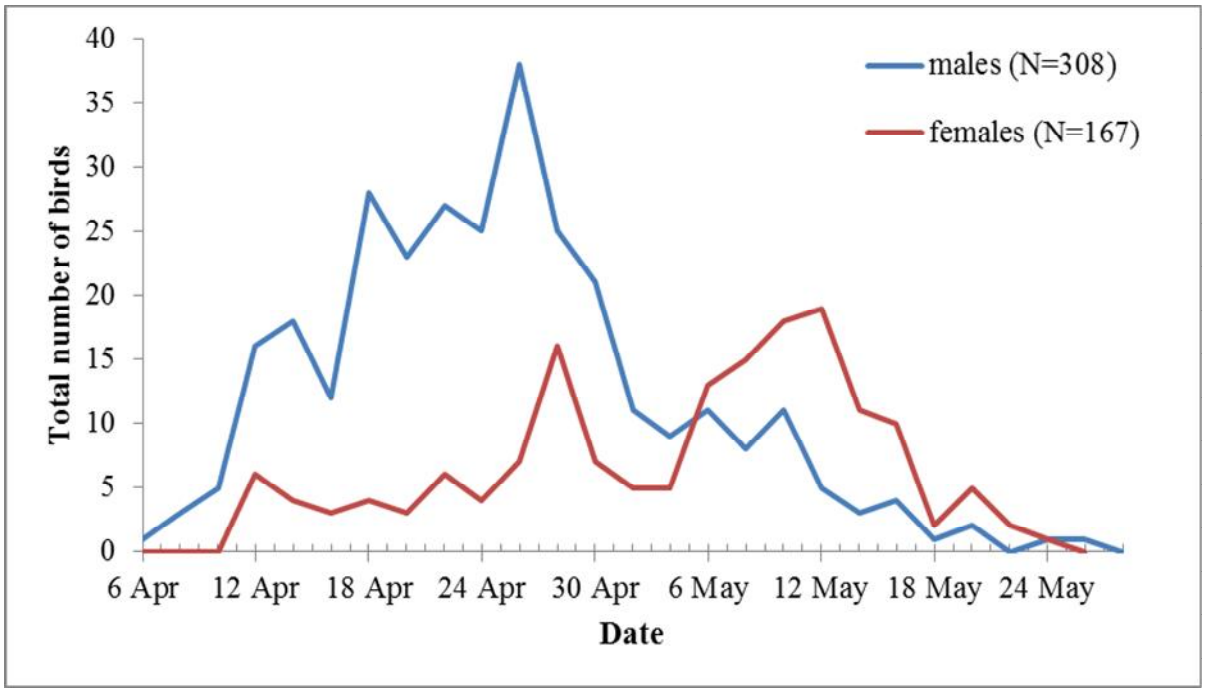

Figure 21. Spring migration dynamics of adult male and female Willow Warblers during six spring migration seasons (2009, 2010, 2012, 2013, 2014, 2015) combined.

In all six seasons combined the median date of migration was 12 days earlier for the males than for the females (Fig. 22), and the difference was highly significant (Mann-Whitney U-test, U = 12956, P < 0.0001).

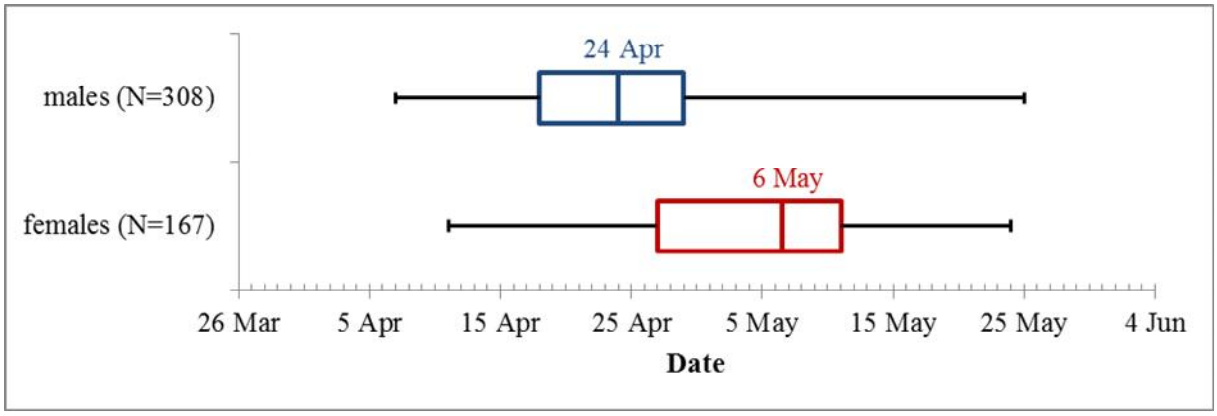

Figure 22. Spring migration phenology of adult male and female Willow Warblers during six spring migration seasons (2009, 2010, 2012, 2013, 2014, 2015), combined. Red boxes – 25-75 % of females, blue boxes – 25-75 % of males, vertical lines and dates – median dates of migration, horizontal lines – first and last occurrence, N – sample size.

I also compared migration timing of males and females in different spring seasons (Fig. 23). In each season the first recorded migrants were the males. The median dates of migration of the males were in different seasons 1 to 21 days earlier than those of the females. Migration timing of females did not differ significantly among seasons (Kruskal-Wallis test, $H_5 = 13.5$, P = 0.142). Migration timing of males differed between some seasons (Kruskal-Wallis test, $H_5 = 28.7$, P = 0.048): in spring 2009 males migrated earlier than in 2010 and 2015 (results of post-hoc tests at Fig. 23).

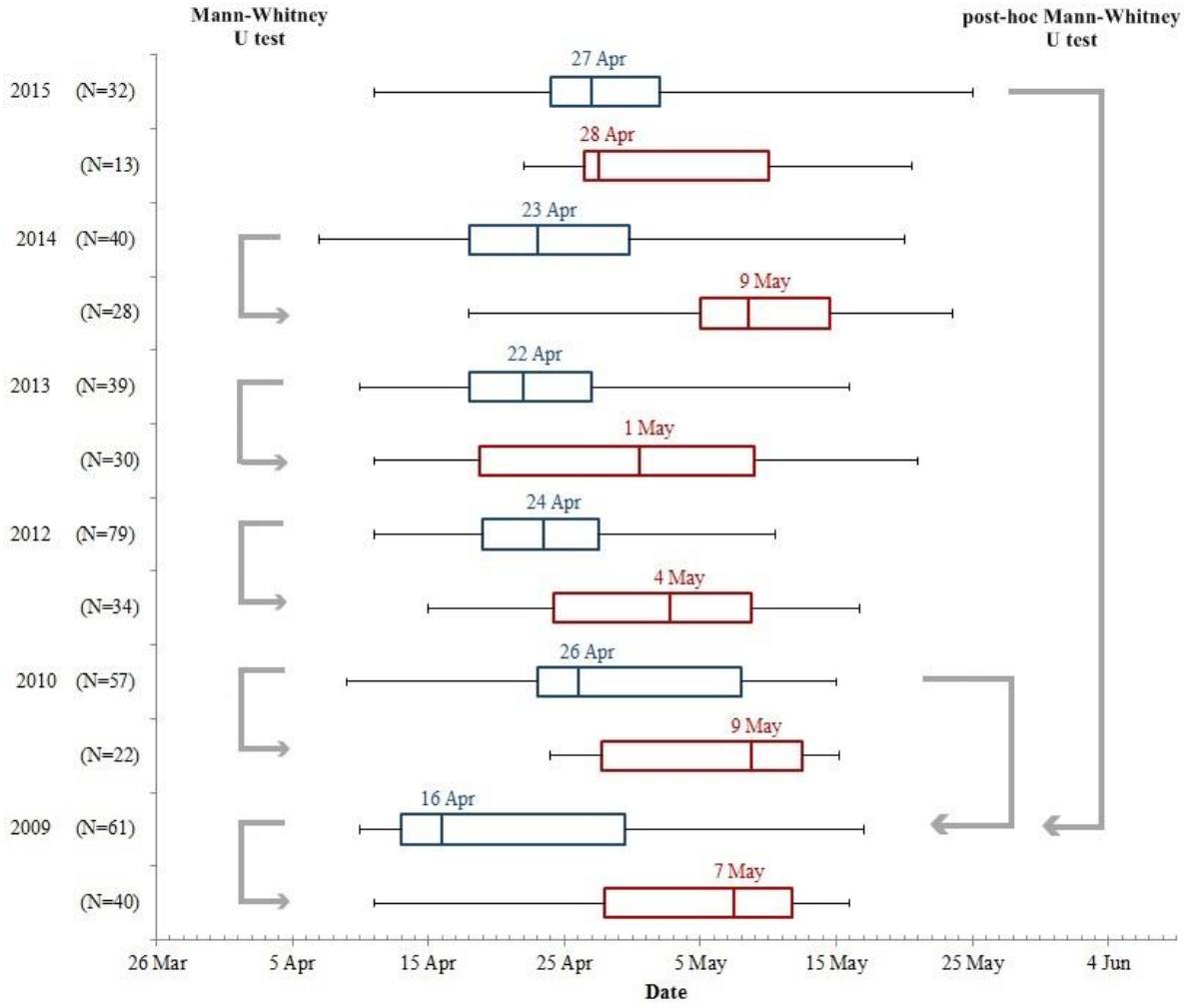

Figure 23. Migration timing of adult Willow Warblers for six spring migration seasons (2009, 2010, 2012, 2013, 2014, 2015). Red boxes – 25-75 % of females, blue boxes – 25-75 % of males, vertical lines and dates – median dates of migration, horizontal lines – first and last occurrence, N – sample size, grey arrows – significant difference between the marked groups ($P < 0.05$) by Mann-Whitney U test.

4.3.2 Spring migration timing of Chiffchaff

I analysed timing of spring migration for 481 morphologically and genetically sexed Chiffchaffs, which were 85 % of all individuals captured during the six spring migration seasons (Appendix p. II, Tab. A2). In six seasons combined the earliest male occurred on 25 March, and the first female occurred 3 days later (Fig. 24). The first small peak of males' migration occurred on 26 March, and the second, main and extended peak occurred between 31 March and 24 April. The first small peak of females' migration occurred on 11 April, and the main extended peak from 15 April to 1 May. The latest males and females were recorded on 24 and 25 May, respectively (Fig. 23).

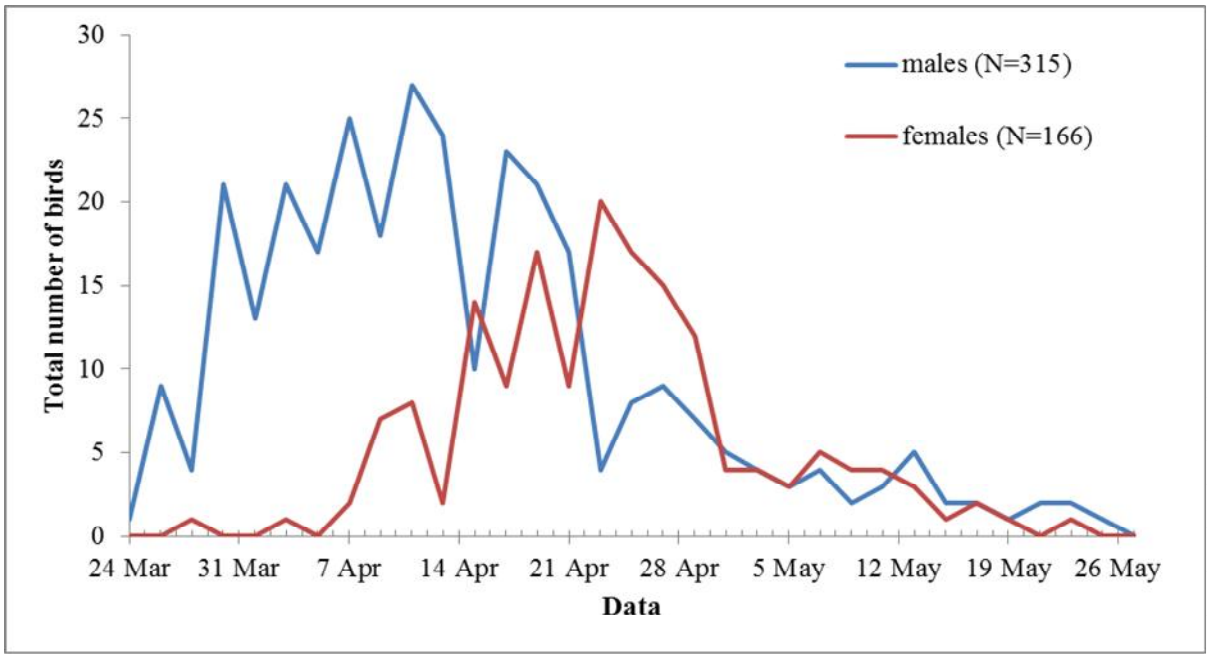

Figure 24. Spring migration dynamics of male and female Chiffchaffs during six spring migration seasons (2009, 2010, 2012, 2013, 2014, 2015), combined.

In all six seasons combined, the males migrated in spring on average 12 days earlier than females (Fig. 25), and the difference between the median dates of migration was highly significant (Mann-Whitney U-test, U = 13038, P < 0.0001).

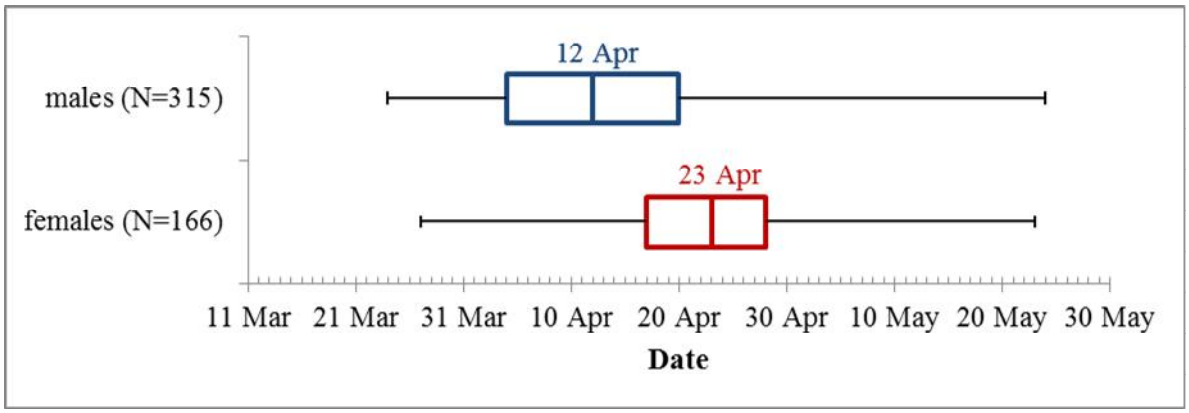

Figure 25. Spring migration phenology of adult male and female Chiffchaffs during six spring migration seasons (2009, 2010, 2012, 2013, 2014, 2015), combined. Red boxes – 25-75 % of females, blue boxes – 25-75 % of males, vertical lines and dates – median dates of migration, horizontal lines – first and last occurrence, N – sample size.

I also compared migration timing of males and females among the different spring seasons (Fig. 26). The first recorded migrants in each season were the males. The median dates of arrival of males were earlier than those of females, and differences between the sexes were significant. In spring 2015 females migrated significantly later than in 2014, 2013, 2010 and 2009 (Kruskal-Wallis test, $H_5 = 28.9$, $P < 0.0001$; results of post-hoc tests at Fig. 26). Migration timing of males differed among most seasons (Kruskal-Wallis test, $H_5 = 28.9$, $P = 0.00002$; results of post-hoc tests at Fig. 26). The males migrated earliest in 2010 and the latest in 2015, on average.

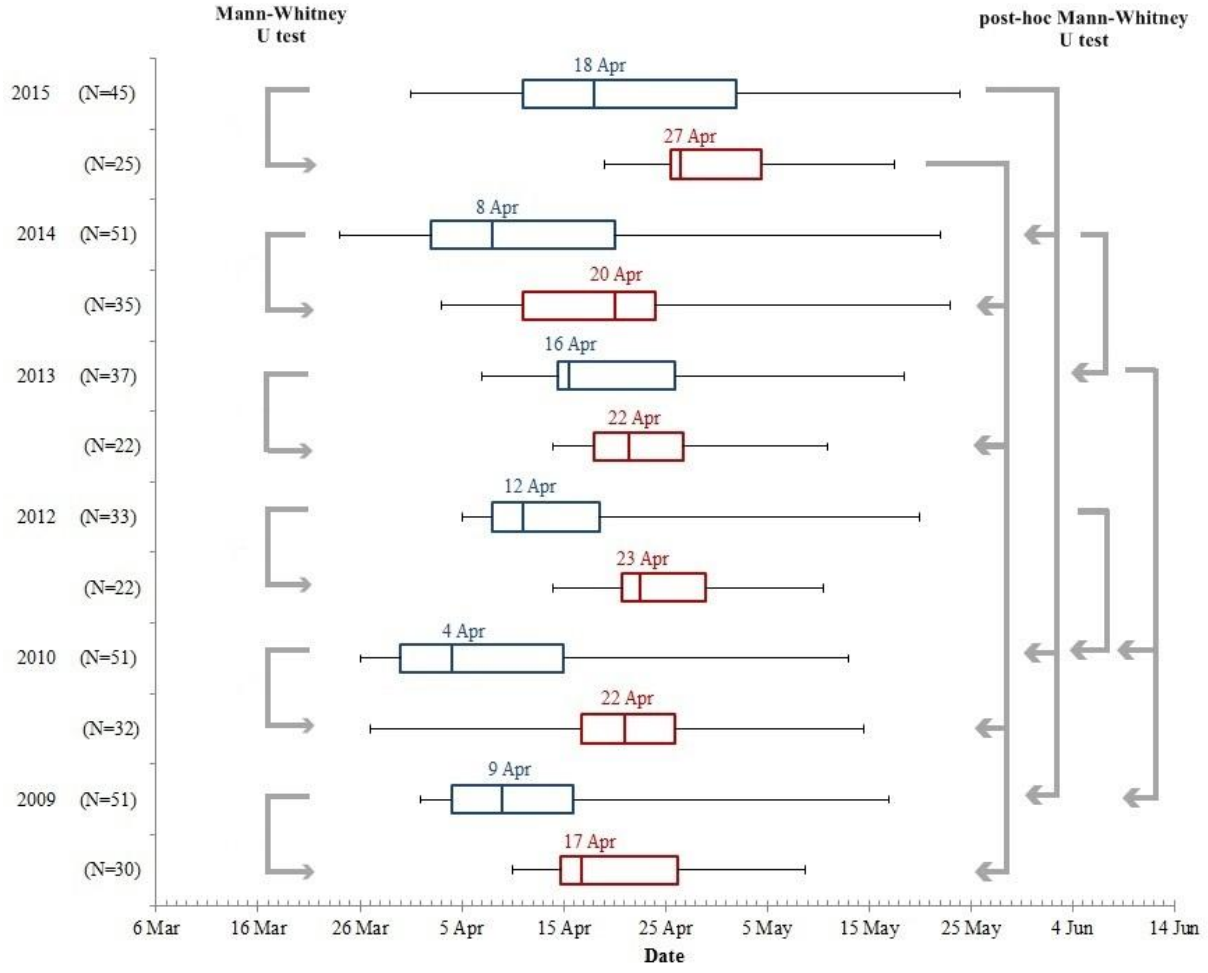

Figure 26. Migration timing of male and female Chiffchaffs for six spring migration seasons (2009, 2010, 2012, 2013, 2014, 2015). Red boxes – 25-75 % of females, blue boxes – 25-75 % of males, vertical lines and dates – median dates of migration, horizontal lines – first and last occurrence, N – sample size, grey arrows – significant difference between the marked groups ($P < 0.05$) by Mann-Whitney U test.

4.3.3 Relationship of spring migration timing of Chiffchaffs with average daily temperatures

To explain some of the differences in migration timing between seasons and sexes, I compared changes in daily ringing numbers of Chiffchaffs with changes of average daily temperature measured at the weather station in Łeba, close to the ringing site, during six spring migration seasons (2009, 2010, 2012, 2013, 2014, 2015). I did not conduct a similar analysis for Willow Warblers because their arrival dates differed significantly only for one extreme season (spring of 2009) from all the other years (Fig. 23).

In spring 2009 temperatures in second half of March mostly fluctuated between 0 °C and 5 °C (Fig 27). The first longer period of temperatures above 5 °C (2–5 April) coincided with the highest peak of arriving males, which continued to be abundant until 20 April. Females started appearing on the days when average temperature stabilised above 5 °C (8–18 April), disappeared again during a short drop of temperature below 5 °C (19–22 April), and then continued arriving until 9 May, when temperatures were above 5 °C.

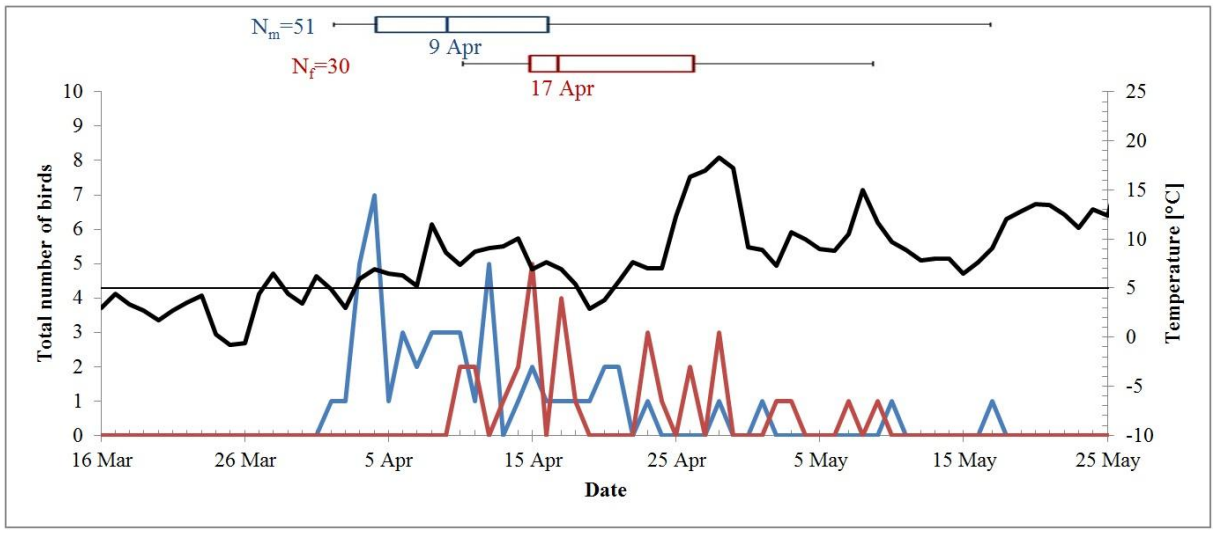

Figure 27. Average daily temperature (black line) and migration dynamics of male (blue line) and female (red line) Chiffchaffs during spring 2009. Thin horizontal line indicates treshold temperature of 5 °C. Box and whisker plots: red boxes – 25-75 % of females, blue boxes – 25-75 % of males, vertical lines and dates – median dates of migration, horizontal whiskers – first and last occurrence, N – sample size.

Spring of 2010 was marked by an early peak of high average temperatures around 15 °C on 25–27 March (Fig. 28). The highest peak in numbers of arriving males and occurrence of a single early female coincided with this early rise in temperatures. The second highest peak of captured males coincided with the next, somewhat lower rise in temperature (29–31 March), after which males continued arriving until 16 May. Median date of males' arrival was earliest of all seasons. In the first half of April temperature rarely went above 5 °C. Females arrived in small numbers after 6 April, with two higher peaks (5 individuals) occurring near the days with temperatures well above 5 °C (18 and 25 April).

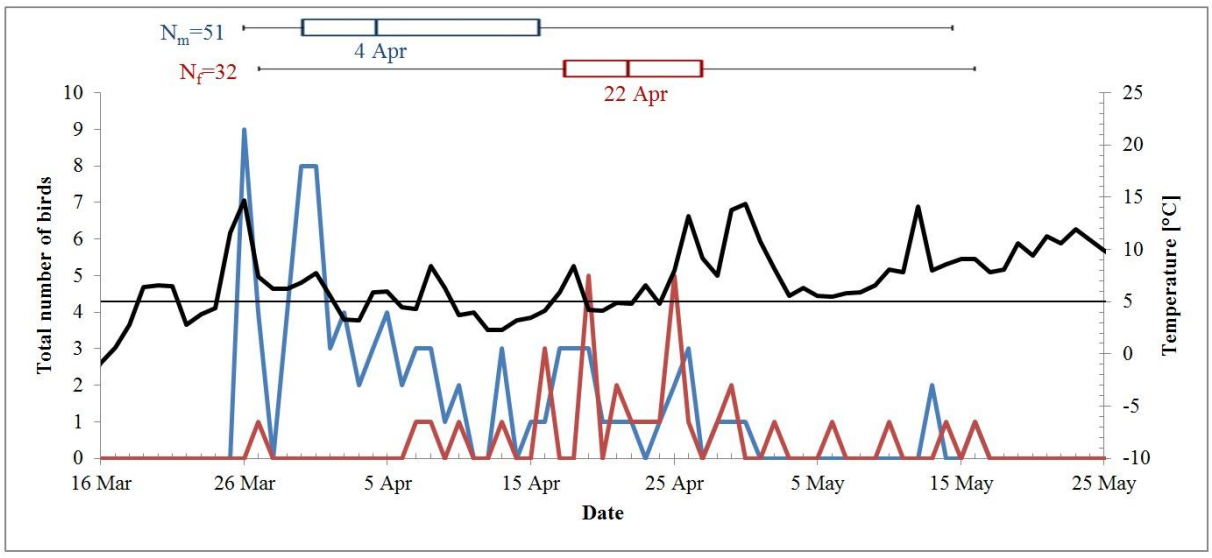

Figure 28. Average daily temperature (black line) and migration dynamics of male (blue line) and female (red line) Chiffchaffs during spring 2010. Thin horizontal line indicates treshold temperature of 5 °C. Box and whisker plots: red boxes – 25-75 % of females, blue boxes – 25-75 % of males, vertical lines and dates – median dates of migration, horizontal whiskers – first and last occurrence, N – sample size.

In spring 2012 males appeared late in the season, and their first and the highest peak occurred during a prolonged period of low average temperature of around 2 °C (2 – 8 April, Fig. 29). The second highest peak in arrival of males coincided with a sudden rise in temperature above 10 °C (9 – 12 April), after which the increases in numbers of captured males followed peaks in temperature. Females started arriving constantly after average temperature stabilised above 5 °C.

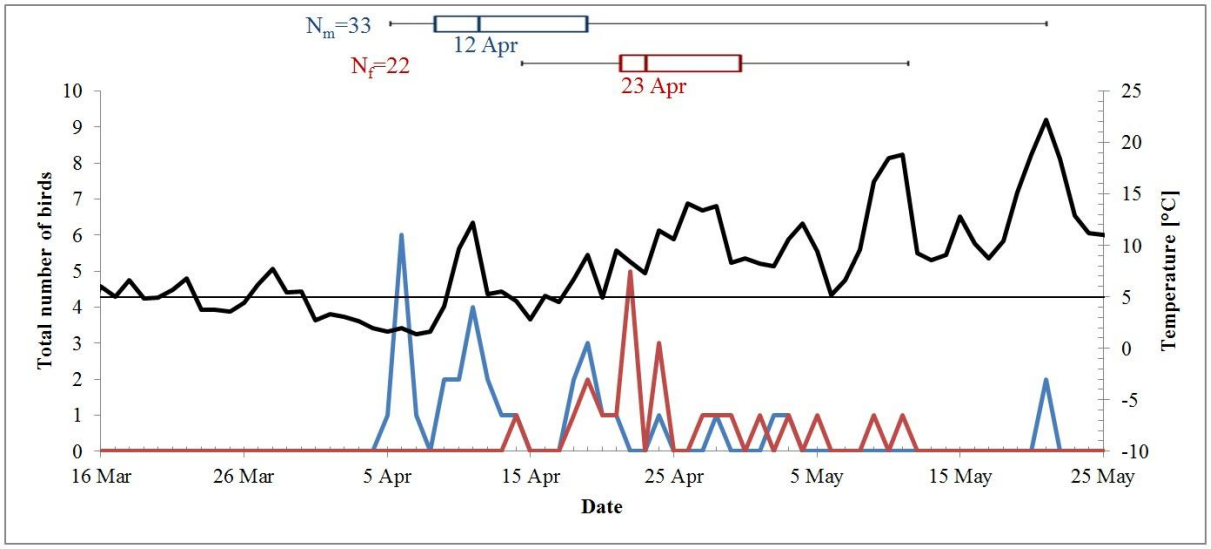

Figure 29. Average daily temperature (black line) and migration dynamics of male (blue line) and female (red line) Chiffchaffs during spring 2012. Thin horizontal line indicates treshold temperature of 5 °C. Box and whisker plots: red boxes – 25-75 % of females, blue boxes – 25-75 % of males, vertical lines and dates – median dates of migration, horizontal whiskers – first and last occurrence, N – sample size.

Beginning of spring 2013 was marked by unusually long period of low average temperatures (0-2 °C) which lasted until 10 April (Fig. 30). First male arrived on 7 April, 2-14 days later than in the other five seasons, and the median date of males' arrival was also later than in most seasons (Fig. 26). The highest peaks in arrivals of both males and females (11–19 April) coincided with first peaks in temperature above 5 °C. Later occurrence of both sexes followed peaks in average temperature.

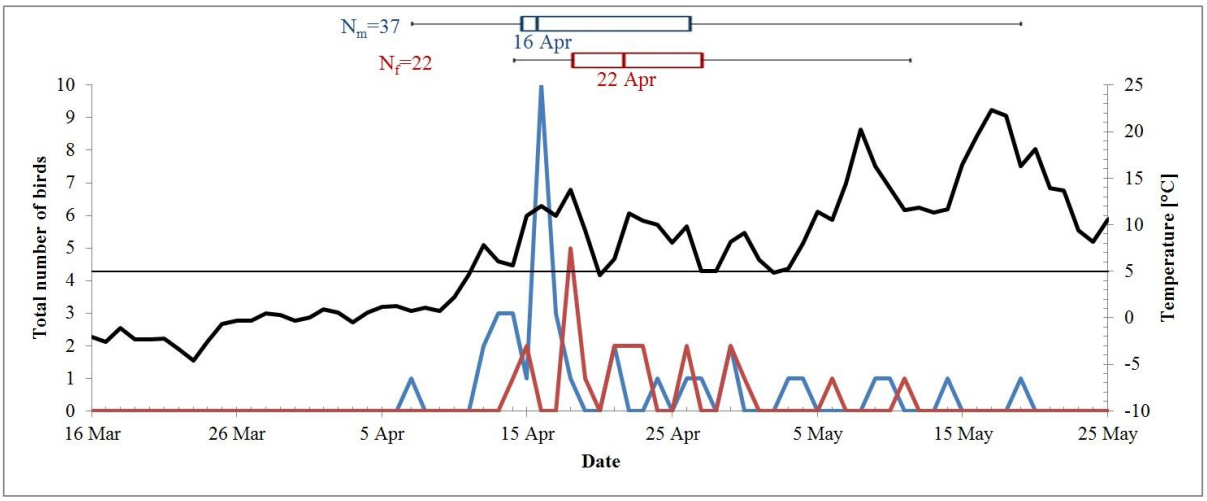

Figure 30. Average daily temperature (black line) and migration dynamics of male (blue line) and female (red line) Chiffchaffs during spring 2013. Thin horizontal line indicates treshold temperature of 5 °C. Box and whisker plots: red boxes – 25-75 % of females, blue boxes – 25-75 % of males, vertical lines and dates – median dates of migration, horizontal whiskers – first and last occurrence, N – sample size.

The start of migration season of 2014 was warm, with average daily temperatures in the second half of March between 3 °C and 10 °C (Fig. 31). The first male arrived on 24 March, which is 2-14 days earlier than in the other five seasons. The first two higher peaks in arrivals of males coincided with days with temperature of 5 °C (28–31 March and 1–4 April). The highest peaks in arrivals of both males and females coincided with the first higher peak in teperature in April (8-12 °C, 5–9 April). Later smaller peaks in numbers of both sexes followed peaks in temperature.

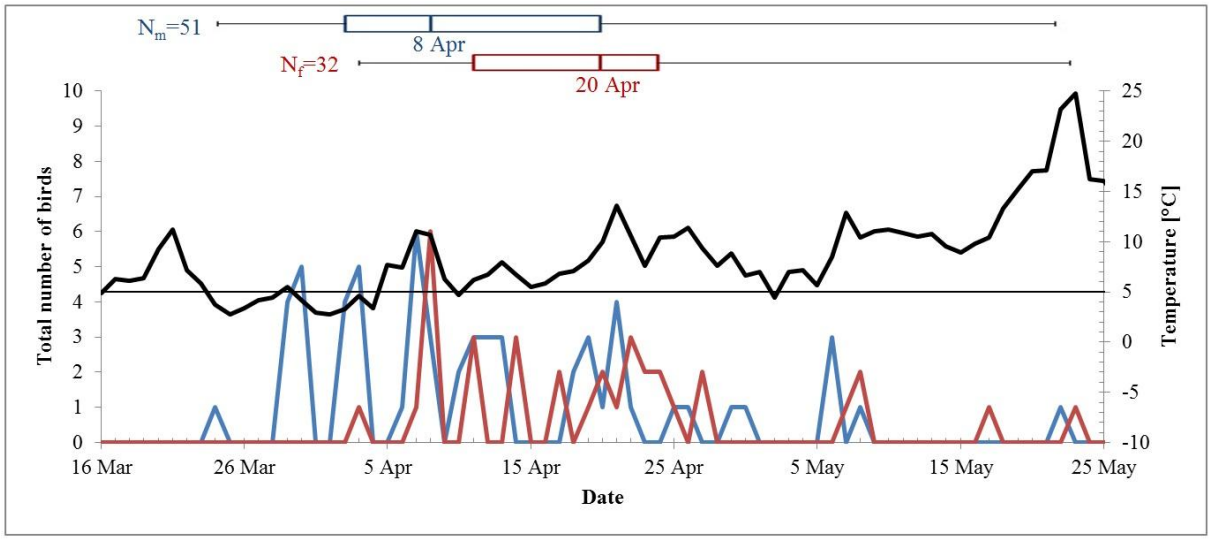

Figure 31. Average daily temperature (black line) and migration dynamics of male (blue line) and female (red line) Chiffchaffs during spring 2014. Thin horizontal line indicates treshold temperature of 5 °C. Box and whisker plots: red boxes – 25-75 % of females, blue boxes – 25-75 % of males, vertical lines and dates – median dates of migration, horizontal whiskers – first and last occurrence, N – sample size.

Temperature trends in spring 2015 were intermediate among all the seasons (Fig. 32). The first peak in arrivals of males coincided with the first higher peak in temperature in April reaching 13 °C (9–12 April). First female arrived on 18 April, 3-24 days later than in the other seasons. The main peak in arrivals of females coincided with the second April peak in temperatures over 10 °C (24–28 April). The median dates of arrival for both sexes were 2-14 days later than in the other seasons.

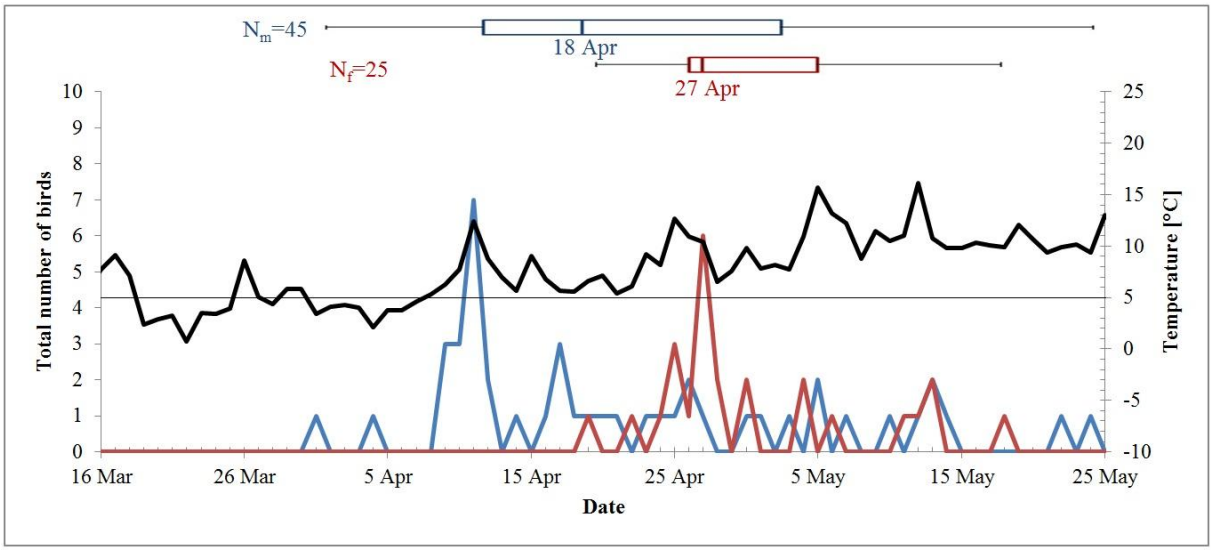

Figure 32. Average daily temperature (black line) and migration dynamics of male (blue line) and female (red line) Chiffchaffs during spring 2015. Thin horizontal line indicates treshold temperature of 5 °C. Box and whisker plots: red boxes – 25-75 % of females, blue boxes – 25-75 % of males, vertical lines and dates – median dates of migration, horizontal whiskers – first and last occurrence, N – sample size.

5. DISCUSSION

5.1 DNA sexing of Willow Warbler and Chiffchaff

Isolation of DNA from blood samples (obtained concentration averaged 35.5 ± 0.2 µg/mL) was more efficient than isolation from feather samples (obtained concentration averaged 7.1 ± 0.4 µg/mL), which confirms the literature comparisons of these two DNA isolation methods (Harvey et al. 2006). I would advocate for continued use of blood samples as a preferred source of DNA for sexing, even though blood sampling requires more equipment, trained personnel and is potentially more stressful for the bird (Bello et al. 2001, Smith et al. 2003, Harvey et al. 2006). Isolation from blood is considerably more reliable, more efficient, simpler and faster way of obtaining ample amounts of DNA material (McDonald & Griffith 2011). While collecting blood samples in the field, I had an opportunity to re-capture several individuals whose blood was collected earlier the same day or several days before. None of the birds appeared to be in bad condition, and had no visible signs of punctuation on the wing or brachial vein. This observation is supported by a number of more extended studies which found no negative effects of blood collection on overall fitness of the sampled birds (Dufty 1988, Hoysak & Wheatherhead 1991, Sheldon et al. 2008). However, I would still advise to sample feathers instead of blood in special cases in which individuals should be released upon capture as soon as possible, e.g. birds in a visibly bad condition, or with an incubation patch, or caught on days with especially cold and/or rainy weather.

Molecular sexing with the use of primers P2/P8 (Griffiths et al. 1998) failed to produce results for 8 % (N=10) of samples from Willow Warblers and 16 % (N=9) of samples from Chiffchaffs. Several studies have reported both neutral and evolutionary favourable genetic variation present in intron regions of chromo-helicase-DNA gene (CHD), to which P2/P8 primers are designed to bind (Dawson et al. 2001, Jarvi and Farias 2006, Toms et al. 2012). Given that the samples used in this study originate from birds that belong to a number of different populations, it is possible that sampled individuals exhibit polymorphisms in intron regions of CHD gene, which makes the P2/P8 primer binding and overall DNA amplification vary in efficiency. The remaining 10 samples from Willow Warblers and 4 of the samples from Chiffchaffs were successfully amplified with the use of more specific F2/R1 primer pair (Bantock et al. 2008). F2/R1 primer pair binds to the intron region of the gene for avian mitochondrial ATP-synthase α-subunit (ATP5A1), and was initially designed for

analysis of damaged and old DNA samples, as a more efficient alternative to P2/P8 primer pair (Bantock et al. 2008). Although amplification by F2/R1 primer pair produced results for most of the samples which could not be amplified by P2/P8 primer pair, I would still not recommend primarily using F2/R1 primer pair if the intended amplicon separation method is agarose gel electrophoresis. F2/R1 primer pair produces shorter amplicons (250-300 bp) than P2/P8 primer pair (300-400 bp), and there is less difference in molecular weight between female-specific and male-specific amplicons. Amplicons produced with the use of F2/R1 primer pair are therefore more difficult to separate by agarose gel electrophoresis. Using a denser agarose gel and higher voltage during electrophoresis improved the separation and enabled me to successfully analyse the amplicons. However, amplicons produced with the use of P2/P8 primer pair were still easier to separate and analyse by agarose gel electrophoresis. I would therefore suggest using F2/R1 primer pair for the analysis of difficult samples for which standard primer pair P2/P8 did not produce results. Alternatively, a more sensitive separation method (e.g. polyacrylamide gel electrophoresis) should be used to separate F2/R1 primer pair amplicons.

Five Chiffchaff DNA samples could not be amplified regardless of the primers used. Four of those samples contained considerably lower concentrations of DNA than others (possibly due to a mistake in isolation process), which is the most probable reason for unsuccessful PCR amplification.

5.2 Morphological sexing of Willow Warbler and Chiffchaff

5.2.1 Sexual size dimorphism in Willow Warbler and Chiffchaff

The present study revealed significant differences in body size between the sexes in Willow Warbler and Chiffchaff, with males being the larger sex in both species. Wing length was the most pronounced sexually dimorphic feature, which confirms the previous research on morphology of these species (Williamson 1967, Norman 1983, Tiainen & Hanski 1985, Svensson 1992, Ellrich et al. 2010). Adult and immature males of Willow Warbler were significantly larger than females in three measurements and males of Chiffchaff were significantly larger than females in almost all of the considered morphological features. According to Tiainen (1982), size dimorphism is most likely a result of Darwinian sexual selection favouring larger males, which are more successful at defending a good quality territory and obtaining a mate than smaller ones. Studies by Tiainen (1982) and Ebenman and

Nilsson (1981) imply that territorial system of both species follows an ideal despotic distribution model, meaning that stronger residents of more suitable habitat force newcomers to choose a less favourable one. During such conflicts larger size provides an advantage, and it might be one of the reasons why both species evolved to be sexually dimorphic.

Differences in wing length are often interpreted together with other features of flight apparatus, most commonly wing shape. There were no sex differences in the indices that reflect the wing shape observed in either of the age groups of Willow Warbler. In immatures, these results correspond with observation by Tiainen & Hanski (1985). The results for adults, however, are opposite to those of all of the previous studies, which consistently report significantly more rounded wing shape in adult females of Willow Warbler than in males (Tiainen & Hanski 1985, Hedenström & Pettersson 1986, Nystörm 1990). According to Hedenström & Pettersson (1986), longer, more pointed wing enables males to fly faster and reach the breeding sites earlier than females, to establish their territory. Dimorphism in wing shape was also correlated with differential parental roles (Blondel et al. 2002). As females do most of the nest building and offspring care, they profit from the increased manoeuvrability provided by a shorter, more rounded wing (Nystörm 1990). A lack of significant difference in wing shape between sexes in Willow Warbler in my study is therefore unusual, and probably should be attributed to the insufficient sample size of adult females used in the analysis.

Significant sex differences in wing shape indices I_E (increasing with higher wing asymmetry, Busse 1967) and I_H (increasing with higher wing asymmetry and pointedness, Hołyński 1965) were observed in Chiffchaff, and indicated more asymmetrical and pointed wing in males. This result is in contrast with the study by Tiainen & Hanski (1985), who found no significant difference between wing shapes in male and female Chiffchaffs. The mentioned study, however, describes significant differences in the wing shape between the two subspecies of Chiffchaff, where *P. c. collybita* has more symmetrical and less pointed wings than *P. c. abietinus*. The difference is explained by *P. c. abietinus* being a long-distance migrant, as opposed to *P. c. collybita*, and therefore having pointed wing more suitable for long distance migration (Gaston 1974, Hansson et al. 2000). The subspecies of Chiffchaff were not noted during fieldwork conducted for this study, but both of these subspecies are known to migrate through Poland. Furthermore, values for wing asymmetry index I_E recorded in my study for both sexes of Chiffchaff had unusually large standard deviation from the mean, which suggests that the sample might include two populations (even subspecies) with different distributions of I_E values. The observed difference in the wing shape between sexes

might therefore be a result of uneven ratio of the two subspecies of Chiffchaff among the samples of males and females. Study by Catry et al. (2005), however, might support the lack of difference in wing shape between sexes observed by Tiainen & Hanski (1985). Catry et al. (2005) proved the differential migration in Chiffchaffs, with females wintering further south and starting spring migration later than males. It is possible that the potential evolution of a more pointed wing in females, which would be beneficial because they migrate further, was counteracted by the advantages of a more rounded wing in female parental care (Piotrowska & Wesołowski 1989).

Differences in size between the age groups in Willow Warbler were much more prominent in males than in females. Adult females showed only a significantly larger mean value of P_1P_2 distance, when compared to immature females. This observation is also in conflict with the other studies that showed highly significant difference in wing length between adult and immature females (Tiainen 1982, Norman 1983, Tiainen & Hanski 1985). This is probably, again, a result of small sample of adult females used in the comparison. Immature males were significantly different from the adults in five out of seven compared morphological features. Lower value of wing pointedness index I_B (Busse 1967) observed in immatures indicated less pointed wing shape when compared to adult birds, while shorter P_1P_2 and longer P_1GC measurement implied that the first primary is longer in immatures, which was then confirmed by an additional comparison of the relative length of the first primary between those two age groups. Shorter, more rounded wing with the longer first primary in immatures than in adults is common in passerines which do not change primary feathers in post-juvenile moult (Alatalo et al. 1984, Peréz-Tris & Tellería 2001). Widely accepted explanation for this difference is that wing shaped this way enables bird to achieve better manoeuvrability, helping young, inexperienced individuals to avoid predators and collect food (Alatalo et al. 1984). By moulting later into the longer and more pointed wing, birds lose some of their manoeuvrability, but are in turn able to fly faster and more efficiently, which is a highly favourable trait for a long-distance migrant such is Willow Warbler (Peréz-Tris & Tellería 2001). Longer Kipp's distance (distance between wing tip and the first secondary) in immature Willow Warblers than in adults is probably not an adaptation, but possibly a result of immatures' secondary feathers still growing during the time that the measurements were taken during the post-breeding season.

Males of Chiffchaff differed from females in seven out of eight morphological features analysed, which suggested more pronounced morphological sexual dimorphism when

compared to Willow Warblers. This is additionally supported by values of Storer's dimorphism index, which were higher in Chiffchaff than in Willow Warbler for all of the morphological features observed. This might reflect a real difference between the species (Tiainen 1982). However, this could also be explained by a possibility that the male and female samples of Chiffchaffs contained unequal ratio of two morphologically distinct subspecies *P. c. collybita* and *P. c. abietinus* (Tiainen & Hanski 1985, Hanski & Tiainen 1991, Hasson et al. 2000), which might increase the apparent differences between sexes. The ratio of Willow Warbler subspecies (*P. t. trochilus* and *P. t. acredula*) in the analysed sample might also affect the morphological comparison between the sexes, but probably to a lower extent. These subspecies, both long-distance migrants, are not as morphologically different as subspecies of Chiffchaff (Tiainen & Hanski 1985, Hanski & Tiainen 1991, Bensch et al. 1999, Hasson et al. 2000). Additionally, the extent of the sexual dimorphism in Willow Warbler might be limited by some factors which are not affecting Chiffchaff. Nyström (1997) argues that the body condition of the bird may be as important as the size, and smaller males tend to have higher feeding efficiency due to better manoeuvrability. Smaller individuals with better body condition were observed successfully defending their territories against larger, but undernourished newcomers. Tiainen (1982) suggests another factor limiting selection for larger males of Willow Warbler – the current size of *Phylloscopus* warblers seems to be relatively safe from predation, because they are small and they offer low energetic profitability to a predator. This would probably change if they were to evolve to a larger size, so it might be one of the limits to their size increase.

5.2.2 Sexing of Willow Warbler and Chiffchaff based on wing length

The present study revealed the wing length as the best single measurement to separate sexes of both species, which corresponds with the previous studies using morphological features to sex Willow Warblers and Chiffchaffs (Tab. 8, Tab. 9).

Willow Warblers caught in spring 2015 were the most uniform group observed, as they consisted of fully grown individuals which all went through complete moult at the winter quarters, and had fresh feathers when caught while migrating to their breeding grounds (Svensson 1992, Jenni & Winkler 2011). In adult Willow Warblers wing length did not overlap between sexes. Females of immature Willow Warblers caught in autumn 2014 had wider ranges of wing lengths when compared to adults, while the range of wing lengths in immature males was shifted towards shorter wings when compared to adults. This could be explained by wings of immatures still growing, and thus shorter than in adults, but this pattern

should then occur in both sexes. My findings partially oppose previous comparisons of age groups in Willow Warbler, which showed a shift in range toward shorter wing lengths in immatures in both sexes, rather than only in males (Norman 1983, Tiainen & Hanski 1985). One of the reasons for this observation might be the small sample sizes of adult females, which inadequately represent the diversity of the studied group of populations. Another reason might be inaccurate ageing of individuals, which results in a mixed sample of adults and immatures. This study showed that adult males had on average 1.2 mm longer wing compared to immatures, while other studies reported the average of 2 mm difference in favour of adults in both sexes (Norman 1983, Tiainen & Hanski 1985). I therefore suspect that the single immature female with recorded wing length of 68 mm is a mis-aged adult, or a mistake was made during measuring. When this individual is excluded, the overlap between sexes in immatures shifts to wing lengths of 66-67 mm. Because of the small available sample size of adults caught in spring, I was not able to develop as fully fitting sexing criteria as for immature Willow Warblers. In a bigger sample of adults the observed range of overlap between sexes would probably span wing lengths of 67-68 mm, since the wing length of the smallest immature males would probably increase from 66 to 67 mm after their first winter moult.

The analysed group of Chiffchaffs caught in spring was not as uniform as Willow Warblers, as it included both birds that retained their natal primary feathers (immatures) and the ones that moulted primaries (adults). Although there are reported morphometric differences between the age groups in Chiffchaff (Tiainen & Hanski 1985), combining adults and immatures did not seem to have a great effect on my results, as the observed zone of overlap between the sexes was still narrow (wing length of 59 mm). A bigger sample is needed to confirm whether the cautious widening of overlap zone to wing lengths of 58-60 mm was justified.

Sexing criteria based on the wing length I suggested to sex both age groups of Willow Warbler are less efficient, but more accurate, when compared to the criteria from the literature (Tab. 15). None of the sexing methods found in literature were 100 % accurate when applied to Willow Warbler data from this study. Interestingly, all of the previous studies recorded smaller minimal wing lengths for both sexes when compared to present study data. Literature criteria which achieved highest accuracy was the most recent study by Ellrich et al. (2010), which also reports higher minimal values of wing length in both sexes when compared to earlier studies (Williamson 1967, Norman 1983, Tiainen & Hanski 1985).

Table 15. Literature overview of sexing criteria based on wing length for adult and immature Willow Warblers. Source, study location, sexing method used and the proposed wing length-based criteria for identifying females and males (expressed in millimetres), are provided. Efficiency (percentage of birds sexed) and accuracy (percentage of correctly sexed birds) of each criteria was calculated for the sample of DNA-sexed Willow Warblers from the present study.

ADULTS

Source	Location	Sexing method	Females	Males	Efficiency (Accuracy)
present study	N Poland	DNA-sexing	≤ 65	≥ 69	90 % (100 %)
Norman (1983)	NE England	cloacal protuberance, brood patch, behaviour	≤ 65	≥ 67	97 % (97 %)
Tiainen & Hanski (1985)	S Finland	cloacal protuberance, brood patch, behaviour	≤ 65	≥ 67	97 % (97 %)

IMMATURES

Source	Location	Sexing method	Females	Males	Efficiency (Accuracy)
present study	N Poland	DNA-sexing	≤ 65	≥ 69	61 % (100 %)
Norman (1983)	NE England	cloacal protuberance, brood patch, behaviour	≤ 63	≥ 65	92 % (82 %)
Tiainen & Hanski (1985)	S Finland	approximation based on data distribution	≤ 63	≥ 65	92 % (82 %)
Williamson (1967)	England	not listed	≤ 63	≥ 67	82 % (94 %)
Ellrich et al. (2010)	SW Germany	DNA-sexing	≤ 64	≥ 68	75 % (98 %)

Sexing criteria for Chiffchaffs based on wing length suggested by Williamson (1967), Ticehurst (1938) and Svensson (1992), although 100 % accurate, are apparently too imprecise to be efficiently used for sexing (Tab. 16). Two sets of criteria were more efficient in sexing Chiffchaffs than those from present study (Lövei 1983, Geen 1988), and both were developed as an approximation based on wing length distribution in studied populations. The bimodal distribution of wing lengths of Chiffchaffs from six spring seasons covered in this study, however, seems to support my choice of the criteria for this species (Fig. 20).

Table 16. Literature overview of sexing criteria based on wing length for Chiffchaff. Source, study location, sexing method used and the proposed wing length-based criteria for identifying females and males (expressed in millimetres), are provided. Efficiency (percentage of birds sexed) and accuracy (percentage of correctly sexed birds) of each criteria was calculated for the sample of DNA-sexed Chiffchaffs from the present study.

Source	Location	Sexing method	Females	Males	Efficiency (Accuracy)
present study	N Poland	DNA-sexing	≤ 57	≥ 61	78 % (100 %)
Geen (1988)	SE England	approximation based on data distribution	≤ 59	≥ 60	100 % (100 %)
Lövei (1983)	N Hungary	approximation based on data distribution	≤ 58	≥ 61	86 % (100 %)
Tiainen & Hanski (1985)	S Finland	cloacal protuberance, brood patch, behaviour, approximation based on data distribution	≤ 61	≥ 63	86 % (73 %)
Ticehurst (1938)	C and W Europe	not available	≤ 56	≥ 62	59 % (100 %)
Svensson (1992)	N and NE Europe	not listed	≤ 58	≥ 64	53 % (100 %)
Williamson (1967)	C and W Europe	not listed	≤ 56	≥ 63	25 % (100 %)

This comparison shows the importance of re-evaluation of the old criteria and developing of new criteria, for accurate and efficient morphological sexing of specific populations of both Willow Warbler and Chiffchaff. Approximation based on distributions of wing lengths might provide satisfactory results in studies which do not aim to sex individual birds (Catry et al. 2005), but in studies where higher precision is required approximations are not acceptable (Nyström 1997). Although there is a potential for improving the criteria proposed in this study by analysis of a larger sample of DNA-sexed birds, they still have numerous advantages over the criteria found in literature, in their current form.

5.2.3 Sexing of Willow Warbler and Chiffchaff based on body size coefficient (PC1)

Sexing criteria based on best body size coefficients (PC1) obtained by Principal Component Analysis (PCA) were more efficient in sexing of both Willow Warblers and Chiffchaffs, when compared to criteria based only on the wing length.

In PCA, larger samples tend to minimize probability of errors, maximize the accuracy of population estimates and increase the generalizability of results (Osborne & Costello 2004). Samples of size N > 300 are considered acceptable for PCA (Comrey & Lee 1992),

and therefore I calculated PC1 for Chiffchaffs (N=355), immature (N=1031) and adult (N=875) Willow Warblers from all of the analysed seasons combined. The criteria for sexing was determined based on PC1 values of the DNA-sexed birds, which makes it not only population specific, but also sample specific. In order for the PCA method of sexing to be applicable, a sample of measurements of already sexed birds must be available to be analysed along with the data for the birds of unknown sex. The sizes of combined samples of sexed and unsexed birds used in this study are most probably large and diverse enough so that if additional measurements of individuals from the same mixture of populations were added, the PC1 based criteria for sexing remain the same.

One of the problems of PCA is that, when used for calculating PC1 from W and P_1P_2, it attributes equal importance to both of these variables. Normally, wing length should be given an advantage over P_1P_2 distance in determining sex of the bird, as it more significantly differs between sexes. Because of these equal weights attributed to both W and P_1P_2 in PC1, a proportion of birds which could easily be sexed using the wing length-based criteria fell into the overlap range between the sexes when PC1-based criteria was used. Another effect of this equal treatment of variables by PCA occurred in these few birds in which application of the PC1-based criteria and the wing length-based criteria gave different results. This resulted in individuals of the female-sized wings and disproportionately large P_1P_2 distances being allocated as males by PC1. This, however, occurred only for 0.004 % of the total analysed sample and is most probably result of an error in one of the measurements.

PC1-based criteria for sexing Willow Warblers and Chiffchaffs improved the sample size of sexed individuals when used together with the wing length-based criteria. Given that the quality of PC1 sexing criteria relies heavily on the distribution of the sample of DNA-sexed individuals, it would be advisable to increase the sample size of DNA-sexed birds from spring to confirm and/or improve the proposed criteria. Distributions of PC1 values of DNA-sexed Chiffchaffs and adult Willow Warblers follow the distributions of the large samples of unsexed birds, suggesting that the proposed criteria is probably justified.

Measurement-based sexing of Chiffchaffs and Willow Warblers is most successful when both criteria based on PC1 and criteria based on wing length are used together. I would therefore conclude that PCA does not provide the most efficient criteria for sexing of these species, since its results need to be improved with the use of a measurement that was already included in the analysis. Better results could be achieved using a statistical method which categorizes measurements by their contribution in separation of sexes. Ellrich et al. (2010),

for example, succeeded in sexing 89 % of immature Willow Warblers applying a forward logistic regression with sex determined by molecular analysis as the dependent variable. I have only succeeded in sexing 66 % of immature Willow Warblers with the use of criteria based on PC1, which is a considerably lower efficiency. Use of PCA, however, is more justified when an additional, third measurement is included into PC1 calculation. Criteria based on PC1 ($W+P_1P_2+S$) showed highly promising results in sexing both Willow Warbler and Chiffchaff, but could not be assessed further because tarsus measurement (S) was not taken in the past and thus the sample was too small. I would therefore suggest further evaluation of these criteria, after increasing the sample of measurements of tarsus length in both species.

5.3 Spring migration timing of Chiffchaffs and Willow Warblers

5.3.1 Effect of subspecies mixture on seasonal differences in migration timing

Combined data from six spring migration seasons showed that the passage of Willow Warblers at Bukowo-Kopań station typically lasts from beginning of April till the end of May. The passage of Chiffchaffs lasts from the end of March till the end of May. Reason for the migration passage of Chiffchaff to start earlier but end at the same time as in Willow Warbler might be the occurrence of two subspecies of Chiffchaff, which cover different distances during migration (Hansson et al. 2000). Although long-distance migrants often leave their wintering quarters earlier and fly faster than the short-distance ones, the short-distance migrants still mostly reach the same breeding grounds earlier (Newton 2010). Short-distance migrant Chiffchaff of the subspecies *P. c. collybita* might therefore be passing Europe in spring earlier than long-distance migrant subspecies *P. c. abietinus*. Lövei (1983) found different "migratory waves" when studying autumn passage of Chiffchaffs in Hungary, which contained different ratios of these subspecies. The population mixture of Chiffchaffs might show a similar pattern in the northern Poland, but revealing this requires more detailed research than the current study. Both subspecies of Willow Warblers represented in this study are long-distance migrants and probably therefore their arrival dates are not spanned as much as in Chiffchaff. The long-distance migration is also the reason why during spring passage Willow Warbler occurs in the study area, and elsewhere in Europe, later than Chiffchaff (Operation Baltic, unpublished data, Bakken et al. 2003, Frasson and Hall-Karlsson 2008, Kralj et al. 2013). Some differences in arrival timing, however, still might occur between the

subspecies of Willow Warblers, because the subspecies *P. t. acredula* migrate to more northern breeding grounds and thus probably pass through Europe earlier than *P. t. trochilus* (Berthold 2001). The two high migration peaks in both sexes of Willow Warbler might support this, although additional research is required for reliable conclusions.

The males of both Chiffchaff and Willow Warbler arrived significantly earlier than females. These results agree with the previous studies on breeding ecology and migration of these species (Geen 1988, Carty et al. 2005, Hedlund et al. 2015). The only exception in our study was spring of 2015, unusual in many regards, when the dates of capture did not differ significantly between male and female Willow Warblers. Protandry (earlier arrival of males) in migrant birds is observed often, but its reasons may differ between species (Morbey and Ydenberg 2001, Møller 2004, Kokko et al. 2006). Willow Warblers that arrive at their breeding grounds earlier in the season have an advantage in choosing a better quality habitat, or finding their territory from previous year(s) unoccupied (Tiainen 1983, Jakobsson 1988, Frostmeier 2002). They are also more likely to protect it successfully against the newcomers due to the "previous ownership" effect – the more time individual spends on a certain territory the more successful it is at defending it, regardless of other factors (Jakobsson 1988). Furthermore, as most of the territorial conflicts occur at the day of the arrival, newcomers are at the considerable energetic disadvantage compared to early settlers (Nystörm 1997). Early arrival of males in Chiffchaff is probably also a result of strong inter-male competition for territories (Tiainen 1982, Piotrowska & Wesołowski 1989, Tiainen & Hanski 1985, Hanski & Tiainen 1991). However, advantages of protandry in terms of breeding biology in Chiffchaff have been studied less than in Willow Warbler. Despite migration timing of male Chiffchaffs varied between seasons and the timing of females did not, males still always arrived earlier than females. Catry et al. (2005) provided strong evidence of sex-differential migration in Chiffchaffs and showed that males winter closer to breeding grounds and begin spring migration earlier than females. Combination of these two factors, departing earlier and wintering closer, might enable male Chiffchaffs to arrive at the breeding grounds before females, even in the years of adverse weather when the start of their migration gets delayed.

The timing of males' passage varied considerably from year to year in Chiffchaff, but less so in Willow Warbler, in which only migration in 2009 was significantly earlier than in two other seasons. Dorka (1966) also observed differences in arrival dates of Chiffchaffs in relation to weather conditions, but found no such differences in Willow Warblers. This could be explained by short-distance migrants being more responsive to the year to year variation in

weather conditions near their breeding grounds than long-distance migrants. Conditions at the wintering grounds near the departure time experienced by short-distance migrants are likely to resemble those at the breeding grounds, enabling birds to adjust their migration timing to the current weather (Berthold 2001, Bridge et al. 2010, Newton 2010). Kokko (1999) suggests that the long-distance migrants evolved a rigid migration timing to achieve the optimal migration schedule, depending on the location and condition at their winter quarters. This is in contrast to the short-distance migrants which need shorter time for return journey and thus might be more flexible and adjust passage to weather within certain time window. Long-distance migrant Willow Warblers are therefore less prone to postpone migration than short-distance migrant fraction of Chiffchaffs, during seasons of adverse weather on route and near the breeding grounds. In spite of the seasonal differences in the first and the mean dates of arrival, Chiffchaffs were captured until the end of activity of the ringing stations each year in the second half of May. The short-distance migrant subspecies *P. c. collybita* might therefore be responsible for the variation in the start and peak of migration each year. The *P. c. abietinus* subspecies, as a long-distance migrant with a more stable migration schedule, could have contributed to the migration dynamics in a similar way each year, and thus affect the observed year-to-year variation less than *P. c. collybita*. This might explain why the observed population of Chiffchaffs, composed of two subspecies that cross different migration distances, manifested migration timing typical of short-distance migrants. However, individuals captured at the end of spring migration season are sometimes local breeders, which did not get captured immediately upon their arrival. This can lead to wrong conclusions about duration of migration passage in different seasons.

5.3.2 Effect of weather on seasonal differences in spring migration timing

To explain some of the differences in migration phenology between the seasons and the sexes in Chiffchaffs, I compared changes in their daily ringing numbers with changes of the average daily temperature. Based on the similarities in arrival dates, I divided the seasons into two groups: 1) years of early arrival, when the median arrival dates of males fell before 10 April, and 2) years of late arrival, when median arrival dates of males fell after 10 April. Within these groups median dates of arrival did not differ significantly, but did differ from the seasons in the other group. I included spring migration seasons 2009, 2010 and 2014 to the "early arrival" group. All these seasons were characterized by stable temperatures of around 5 °C in early spring (second half of March). Spring migration seasons 2012, 2013 and 2015 formed the "late arrival" group. These seasons do not have one common characteristic in

terms of average spring temperatures. Considering that early arrival at the breeding grounds has multiple positive effects on fitness in males (Kokko 1999, Morbey and Ydenberg 2001, Møller 2004), I examined closely the "late arrival" seasons to determine the probable reasons for delayed arrival of Chiffchaffs. Early spring in 2012 was warm, with temperatures over 5 °C, but despite these potentially favourable conditions Chiffchaffs started appearing late in the season (on 5 April). This delay in 2012 might have been caused by conditions in the wintering grounds. Several studies have shown how shortage of food supplies at the wintering sites, in the effect of low temperatures or lack of rainfall, can significantly delay onset of birds' spring migration (Bridge et al. 2010, Studds and Marra 2011, Rockwell et al. 2012). Additionally, low temperatures can affect the rate of pre-migratory fattening due to elevated energy demands associated with living in cold (McWilliamson and Karasov 2005). According to the ringing recoveries (Operation Baltic, unpublished data, Bakken et al. 2003, Frasson and Hall-Karlsson 2008), most of Chiffchaffs of the *P. c. collybita* subspecies passing through northern Poland probably winter in the south-eastern Spain. Average daily temperatures at several meteorological stations in south-east of Spain (Seville, Granada, Valencia, Albacete; https://weatherspark.com) during winters preceding the spring migration seasons covered in this study showed a prolonged period of unusually low temperatures during the first half of February 2012, which did not occur during other winters. Chiffchaffs need approximately 6 weeks to cross the distance from south-east Spain to the Polish coast (Operation Baltic, unpublished data, Bakken et al. 2003, Frasson and Hall-Karlsson 2008). This means they probably start their pre-migratory hyperphagia in early February (McWilliamson and Karasov 2005, Berthold 2001, Newton 2010). Therefore prolonged colds in February 2012 in Spain might have had negative effect on Chiffchaffs' pre-migratory fattening and delayed onset of their migration in 2012. Additionally, winter of 2011-2012 in Spain was exceptionally dry (Guerrero 2012), which also might have had negative influence on food availability at Chiffchaffs' wintering sites. Explaining Chiffchaffs migration delay in 2012, however, includes many assumptions, confirmation of which goes beyond the scope of this study. Early spring of 2013 was characterized by a long period of exceptionally low temperature on the Baltic coast, and first males started arriving late in the season. Soon after the passage started, 50 % of all males recorded that season were captured within 5 days, as soon as the temperature exceeded 5 °C. Since arrival at the breeding site before the increase in temperature and spring green-up usually results in starvation and death, males are prompted to adjust timing of their migration to external weather conditions and spring phenology of the breeding area (Alerstam et al. 2003, Emmenegger et al. 2014, Hahn et al. 2016). Male

Chiffchaffs probably delayed their migration in 2013 until weather conditions, and thus food availability, improved at their stopover locations and breeding sites (Balboltín et al. 2009, Emmenegger et al. 2014, Kölzsch et al. 2015). In spring 2015 temperature was high and stable on the Baltic coast, and there were no prominent periods of low temperatures during winter 2014/2015 in south-east Spain. However, median dates of arrival of both males and females were later in 2015 than in all other seasons. Spring of 2015 was unusual in terms of migrants occurrence as compared to previous seasons at Bukowo-Kopań ringing station, with delay in passage and smaller numbers of ringed long-distance and short-distance migrants alike (Operation Baltic, unpublished data). This major shift in migration phenology of Chiffchaffs and other migrants in 2015 could be an effect of a combined influence of other weather conditions, e.g. temperature, wind and precipitation, at both the wintering site and on their migration route, which requires further analysis.

Although peaks in occurrence of both males and females mostly followed peaks in temperature, females seem to be more selective than males, as they almost never appeared in bigger numbers ($N \geq 3$) on the days when the mean temperature is below 5 °C (Figs. 27-32). This tendency highlights the importance of Bukowo-Kopań as a stopover site. Spring migration occurs under bigger time pressure than autumn migration as there are numerous benefits of early arrival to breeding site (Kokko 1999, Moore et al. 2005, Nilsson et al. 2013). Chiffchaffs migrate mostly during the night, and use daytime to replenish their energy reserves at stopover sites (Ciach 2009). Successful migration involves a trade-off between the number of stopovers and their duration (Alerstam 2011). High quality stopover sites which ensure fast recovery of energy supplies are therefore especially important to birds during spring migration race (Nilsson et al. 2013, Arlt et al. 2015). As discussed earlier, temperature has a direct influence on food availability and metabolism rate of individuals (McWilliamson and Karasov 2005, Newton 2010), and higher temperature usually means higher quality of stopover sites and abundance of insects (Elkins 2004, Marra et al. 2005, Månsson and Hämäläinen 2011). Therefore, bird numbers present at Bukowo-Kopań probably at least partially reflect food condition at the site at the time (Newton 2010). Since the males face higher pressure of early arrival to breeding site (Kokko 1999, Morbey and Ydenberg 2001, Møller 2004) and migrate earlier in the season than the females, when the temperatures are lower and more unstable than later, they are more prone to be forced to use a stopover site during suboptimal temperatures. Females migrate later in the season, when their coinciding stopovers with optimal temperature is probably easier. We can also look at differential migration timing of males and females from the perspective of selective pressures sexes face

at the beginning of the breeding period. Since females are expected to produce eggs soon after their arrival to breeding site, it is highly advantageous for them to arrive in good physiological condition (Sandberg and Moore 1996, Moore et al. 2003). Females are therefore more likely to make a trade-off in favour of good physiological condition over early arrival, and rather arrive later but with more residual nutrient supplies than males (Newton 2010). Sandberg and Moore (1996) hypothesise that females which arrive with greater nutrient reserves are able to start breeding earlier and produce higher quality eggs than those in worse condition. Thus, the preference of females to use stopover sites only in periods of optimal temperature, observed in this study, might be due to expected energy requirements at the breeding site (Sandberg and Moore 1996, Moore et al. 2003).

I did not conduct analogous analysis of migration phenology of Willow Warblers against temperature at the ringing site, because their timing of passage did not vary much among the seasons, as I discussed earlier. Additionally, Willow Warblers arrive at the ringing site later in spring than Chiffchaffs, when the temperature is already high and has less effect on their migration speed and choice of stopover. However, there is potential for long-term study of change in arrival timing of Willow Warblers. Although long distance migrants have been perceived to mostly rely on intrinsic cues to start their migration (Newton 2010), Saino et al. (2007) showed clear connection between amount of rainfall in wintering areas in Africa and subsequent spring arrival dates of nine long-distance migrants, Willow Warbler included. It is possible that the present study did not compare enough seasons to observe such differences. In a later study, Saino and Ambrosini (2010) argue that weather conditions in sub-Saharan western Africa during late winter co-vary with those in Europe during spring, thus allowing long-distance migrants to predict weather conditions at the breeding sites before they start migration. However, the strength of this correlation of weather in western Africa and Europe has declined during the past 25 years, making it harder for migrants to adjust their migration schedules to conditions at the breeding grounds. Therefore, the arrival dates of Willow Warblers might have been more flexible in the past. Climate change seems to be an important factor in changing trends in bird migration (Marra et al. 2005, Møller et al. 2008, Newton 2010, Hüppop & Hüppop 2011). Heldlund et al. (2015) noted long-term phenological shifts in Willow Warblers, with both sexes arriving and breeding approximately 5 days earlier than 22 years ago. Hence, a more long-term study of trends in migration timing might reveal greater differences in migration timing in populations of Willow Warbler that migrate through northern Poland.

6. CONCLUSIONS

The results presented in this study can be summarised as follows:

1. Most Chiffchaffs (84 % of sampled individuals) and Willow Warblers (92 %) were successfully DNA-sexed using the standard primer pair P2/P8. For the problem samples, F2/R1 primer pair and an adjusted separation protocol can improve the results. In total, 100 % of sampled Willow Warblers and 92 % of sampled Chiffchaffs were DNA-sexed using the combination of these two pairs of primers.

2. Both species show distinct sexual dimorphism, with males being the larger sex. Wing length was the most pronounced sexually dimorphic feature. Size dimorphism in these species is probably a result of Darwinian sexual selection favouring larger males, and might also be related to differential migration strategies and parental roles in sexes.

3. The most efficient morphological sexing criteria were based on PC1 combining wing length and P_1P_2 measurement. The most widely applicable criteria for both species were based on wing length, since the P_1P_2 measurement was not available for some of the past data. With the combined use of both of these criteria I was able to sex 90 % of adult Willow Warblers, unsampled for DNA, and 88 % of unsampled Chiffchaffs captured during spring migration seasons 2009-2015.

4. Differential migration in the form of protandry was observed in both species. Males arrived earlier than females in most seasons, except spring 2015, when the dates of arrival of Willow Warblers did not differ between the sexes. Early arrival probably gives males an advantage in competition for mates and better quality territories.

5. The timing of males' spring arrival varied considerably among years in Chiffchaff, but less so in Willow Warbler, pointing to a more rigid migration schedule in the latter species. There were no notable year-to-year differences in timing of passage in females in either species. Literature data suggest that low temperature at the wintering sites or on the migration route may delay spring passage of male Chiffchaffs.

6. The rise in temperature at the stopover site often coincided with the peaks in arrivals in both male and female Chiffchaffs. Higher temperature seems to have a positive influence on the quality of a stopover site, in terms of feeding conditions and food abundance.

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APPENDIX

Table A1. Sample sizes of morphological measurements of Willow Warblers used in the study. Willow Warblers were caught during six autumn (A: 2014, 2013, 2012, 2009, 2008, 2007) and six spring (S: 2015, 2014, 2013, 2012, 2010, 2009) migration seasons, and were divided into three age groups: A – adults caught in autumn or spring, I – immatures, L – birds of unknown age. The measurements included are: W – wing length, P_1P_2 – distance between the first and the second primary feather, K – distance between the longest primary feather and the first secondary feather, P_1GC – distance between the first primary and the longest greater covert, WF – wing formula and S – tarsus length.

Measurement	Age	2014 A	2013 A	2012 A	2009 A	2008 A	2007 A	2015 S	2014 S	2013 S	2012 S	2010 S	2009 S
W	A	16	8	3	25	4	66	45	75	73	131	85	109
	I	480	153	43	330	143	184	-	-	-	-	-	-
	L	133	7	2	15	146	106	-	-	-	-	-	-
	total	**692**	**168**	**48**	**370**	**293**	**356**	**45**	**75**	**73**	**131**	**85**	**109**
P_1P_2	A	16	8	3	19	3	13	45	73	70	130	85	108
	I	469	152	38	284	119	101	-	-	-	-	-	-
	L	132	7	2	14	104	16	-	-	-	-	-	-
	total	**617**	**167**	**43**	**329**	**226**	**130**	**45**	**73**	**70**	**130**	**85**	**108**
K	A	16	8	3	-	-	-	45	75	70	129	85	109
	I	468	151	35	-	-	-	-	-	-	-	-	-
	L	131	7	2	-	-	-	-	-	-	-	-	-
	total	**615**	**166**	**40**	**-**	**-**	**-**	**45**	**75**	**70**	**129**	**85**	**109**
P_1GC	A	16	8	3	19	3	14	45	72	49	89	64	89
	I	472	150	36	295	93	101	-	-	-	-	-	-
	L	131	7	2	14	110	16	-	-	-	-	-	-
	total	**619**	**165**	**41**	**328**	**206**	**131**	**45**	**72**	**49**	**89**	**64**	**89**
WF	A	16	8	3	25	4	57	45	65	58	97	78	101
	I	470	149	39	318	134	175	-	-	-	-	-	-
	L	131	7	2	14	135	97	-	-	-	-	-	-
	total	**617**	**164**	**44**	**357**	**273**	**329**	**45**	**65**	**58**	**97**	**78**	**101**
S	A	-	-	-	-	-	-	45	-	-	-	-	-
	I	-	-	-	-	-	-	-	-	-	-	-	-
	L	-	-	-	-	-	-	-	-	-	-	-	-
	total	**-**	**-**	**-**	**-**	**-**	**-**	**45**	**-**	**-**	**-**	**-**	**-**

Table A2. Sample sizes of morphological measurements of Chiffchaffs used in the study. Chiffchaffs were caught during six spring migration seasons (2015, 2014, 2013, 2012, 2010, 2009), and were divided into three age groups: A – adults from autumn or spring, I – immatures, L – birds of unknown age. Measurements included are: W – wing length, P_1P_2 – distance between the first and the second primary feather, K – distance between the longest primary feather and the first secondary feather, P_1GC – distance between the first primary and the longest greater covert, WF – wing formula and S – tarsus length.

Measurement	Age	2015	2014	2013	2012	2010	2009
W	A	22	12	3	4	10	1
	I	38	81	58	36	98	55
	L	13	23	12	29	28	42
	total	**73**	**116**	**73**	**69**	**136**	**98**
P_1P_2	A	22	12	3	3	7	1
	I	32	77	39	10	87	36
	L	10	23	9	7	25	30
	total	**64**	**112**	**51**	**20**	**119**	**67**
K	A	21	11	3	4	-	-
	I	26	56	33	8	-	-
	L	9	19	8	10	-	-
	total	**56**	**86**	**44**	**22**	**-**	**-**
P_1GC	A	22	12	3	4	8	1
	I	32	77	34	7	87	36
	L	8	22	10	11	25	30
	total	**62**	**111**	**47**	**22**	**120**	**67**
WF	A	20	10	1	4	8	1
	I	26	56	28	8	74	38
	L	9	19	8	10	24	27
	total	**55**	**85**	**37**	**22**	**106**	**66**
S	A	22	-	-	-	-	-
	I	30	-	-	-	-	-
	L	13	-	-	-	-	-
	total	**65**	**-**	**-**	**-**	**-**	**-**

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May 2010 - May 2014 **Ornithological Biodiversity Research Projects** Croatia
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Study of bird population structures and endangered bird species;
Nature Park Grabovača (2014), island of Cres (2013), Dinara Mt. (2012),
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Mar 2011 - Jun 2011 **Undergraduate Research Project** Zagreb, Croatia
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WORK EXPERIENCE

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Participation in NIP Phase II - ornithological biodiversity monitoring;
Experience in point count and transect census methods

Oct 2015 & Sep 2013 — **Raptor Migration Count Batumi** — Batumi, Georgia
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Participation in an ongoing raptor count project;
Experience in raptor identification and migration count

Mar-May 2015 & Sep 2013 — **Ornithological Camp Bukowo** — Bukowo, Poland
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Participation in an ongoing research project on bird migration trends;
Experience in bird banding, mist netting and using wader traps

Mar 2014 - Jun 2014 — **Common Bird Monitoring Project** — Croatia
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Participation in newly established project focused on common bird species;
Experience in point count and transect census methods

Feb 2013 - Mar 2014 — **CRO-Fauna Project** — Zagreb, Croatia
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Standardization of ornithological research data and import into developing database

Oct 2013, Sep 2011 & Sep 2010 — **Ornithological Camp Učka** — Nature park Učka, Croatia
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