



Biased sex-ratio in woodland grouse population of the Tatra National Park, suggested by molecular sexing of non-invasive samples

Anna SANTOREK¹, Tomasz ZWIJACZ-KOZICA², Beata DULISZ³, Dorota MERTA⁴
and Robert RUTKOWSKI¹

¹Museum and Institute of Zoology, Polish Academy of Sciences, Wilcza 64, 00-679 Warszawa, Poland

²Tatra National Park, Kuźnice 1, 34-500 Zakopane, Poland

³Faculty of Biology and Biotechnology, University of Warmia and Mazury in Olsztyn,
Plac Łódzki 3, Olsztyn 10-727, Poland

⁴Department of Ecology and Environmental Protection, Pedagogical University of Kraków,
Podbrzezie 3, 31-054, Kraków, Poland

*Corresponding author email: robertrut@miiz.waw.pl

Abstract: Sex ratio (ratio between the number of male and female individuals in a population) is an important factor contributing to the survival rates of the population in wild. Imbalanced sex ratio is often the cause of diminished survival rates of endangered species. The analysis of DNA collected through noninvasive methodologies effectively allows to monitor populations of rare species or species that are difficult to observe in the wild. Such method was used to determine the sex ratio in populations of the capercaillie (*Tetrao urogallus*) and black grouse (*Lyrurus tetrix*) in the Tatra National Park. We collected over 250 samples (mostly excrement) in the area of the Tatra National Park and identified species via microsatellite genotyping and determined the sex using fragments of the CHD gene. We found that the majority of the samples belonged to male specimens irrespective of the species suggesting a large deviation from an equal sex ratio in wild populations of capercaillie and black grouse in the Tatra National Park. Our results point out the fundamental contribution of genetic assessment of sex ration to monitor the viability of both species in the Tatra National Park

Key words: *Tetrao urogallus*, *Lyrurus tetrix*, sex ratio, genetic sexing, non-invasive sampling

INTRODUCTION

The relative proportion of male to female individuals within a population, the sex-ratio, is one of the most important topics in population ecology (Hardy 2002). The ratio of males and females at conception, at birth and adulthood (defined as primary, secondary and adult sex ratios) has a significant influence on behaviour of individuals and the viability and even survival of the population. According to Fisher (1930), the sex ratio of a viable population should be around 1:1. However, the exact proportion in the cohort of breeding adults depends on several processes, e.g. sex-dependent differences in mortality at birth, sexual maturity, sex-biased and dispersal (Bessa-Gomes et al. 2004). Such processes often result in a significant variation of the relative male and female proportion at the local scale, although these differences may be counterbalancing at the meta-population level. In many species the secondary sex ratio might substantially deviates from a 1:1 ratio. For example, raising male or female offspring might involve differential costs or benefits in parental care (West 2009). In addition social, environmental, genetic and physiological factors all contributes to the gender structure of a population (Navara 2018a). Females might also be able to influence the sex of their offspring at birth (Trivers & Willard 1973, West 2002, Pryke & Rollins 2012). For example, Clout et al. (2002), in an attempt to enhance captive breeding of the polygynous kakapo (*Strigops habroptila*), was able to skew the sex ration towards males through intensive feeding of adult females. When environmental conditions are favourable, females produce more males, and rising them requires more energy. With moderate food resources, females produce offspring in a 1:1

ratio (Robertson et al. 2006). Thus, environmental conditions might affect the sex ratio of a population, which may be of the utmost importance in the case of protected species. Both the mating system and the sex ratio influence the demography and extinction risk of a population (Lee et al. 2011, Navara 2018b). An unequal sex-ratio of the adult cohort might lead to a reduction of the effective population size and consequently decrease the viability of the population (Donald 2007). For example, Lambertucci et al. (2013) suggested that the sex-ratio of released the Andean condor (*Vultur gryphus*) significantly affected the effectiveness of reintroduction and reinforcement programmes.

The sex ratio of a population is often difficult to assess if based only on observational methods. Many bird species show low level of sexual dimorphism, which makes it difficult or even impossible to determine their sex based on morphology. Determining the sex of the chicks is always problematic even in species with adult sexual dimorphism, as features of the sexually dimorphic plumage do not become apparent until sexual maturity. Furthermore, even with pronounced dimorphism in adult birds, the behaviour of males and females might cause difficulties in assessing a true sex-ratio. Often one of the sexes is either more mobile or secretive than the other, leading to incorrect inference of the sex-ratio based solely on observational methods (Théry 1992; Vanderkist et al. 1999, Blackmore et al. 2011). The identification of the sex via genetic methods (molecular sexing) has therefore proved invaluable in the study of wild bird populations (Dubiec & Zagalska-Neubauer 2006). PCR-based methods using sex-linked markers are commonly used for sex determination. Besides the highly conserved genes CHD1W/CHD1Z (Ellegren & Sheldon 1997), also the genetic marker Wpcki has been successfully used for molecular sexing (Hori et al. 2000). The development of molecular methods for sex determination has thus allowed to overcome the intrinsic difficulties due to lack of clear phenotypic differentiation (Segelbacher & Steinbrück 2001, Waits & Paetkau 2005, Regnaut et al. 2006). Moreover, thanks to the use of genetic methods, it is now possible to determine the sex of an individual using non-invasive sampling (feathers, faeces found in the field). In combination with genotyping, molecular sexing allows the determination of sex ratio based on biological material obtained without the need to capture individuals, and is often the method of choice for endangered, elusive or low-density species. Advances in DNA extraction and DNA amplification technology make non-invasive sampling a source of extremely informative material for genetic research (Taberlet et al. 1999, Segelbacher & Steinbrück 2001, Piggott et al. 2004, Broquet et al. 2007, Beja-Pereir et al. 2009, Baumgardt et al. 2013). The effectiveness of sex determination via DNA analysis from non-invasive sampling has been demonstrated for a variety of species and from a variety of biological material such as feathers, hair and droppings (Eggert et al. 2003, Bush et al. 2005, Fontanesi et al. 2008, Baumgardt et al. 2013, Yamashiro et al. 2017).

The aim of this study was to determine the sex ratio in wild populations of two endangered forest grouse living in the Tatra National Park: the capercaillie (*Tetrao urogallus* Linnaeus, 1758) and the black grouse (*Lyrurus tetrrix* Linnaeus, 1758). Mountain forests with a low degree of fragmentation and human disturbance are an important stronghold for the forest grouse in Europe (Storch 2007). The Tatra Mountains represent an important refuge for the capercaillie and black grouse, and observations suggest a higher number of individuals than in other similar areas (Ciach 2016, Rutkowski et al. 2017a, Rutkowski et al. 2017b). In small and threatened populations, the degradation of the habitat, for example due to fragmentation, may cause significant deviations from an equal sex ratio (Helle et al. 1999, Sun et al. 2003). In polygynous species, such as the capercaillie, the risk of extinction is lowest when the adult sex ratio is biased towards females (Bessa-Gomes et al. 2004). The capercaillie and the black grouse are endangered, thus understanding the demographic and sex-ratio of wild populations is fundamental for the protection of both species.

MATERIAL AND METHODS

Sample collection

We collected 100 capercaillie and 166 black grouse samples during field work conducted in 2018 and 2019. All the samples were collected via non-invasive means (261 faeces and 5 feathers). Faeces were stored in a freezer at -22°C until DNA extraction, whereas feathers were kept in paper envelopes and stored in a freezer at -4°C until DNA extraction. Samples come from the Polish side of the Tatra Mountains protected as the Tatra National Park.

Laboratory analysis

We extracted DNA from droppings using the NucleoSpin Soil Kits (MACHEREY-NAGEL) and later GeneMATRIX Stool DNA purification Kit (Eurx), following the manufacturers' protocols. We extracted DNA from feathers using a GeneMATRIX Tissue DNA Purification Kit (Eurx) using the standard protocol of the manufacturer. The detailed procedure for DNA isolation process in order to minimise contamination is described in Rutkowski et al. (2017b) and Santorek et al. (2018).

To identify unique genotypes (thus individuals) among collected samples we conducted microsatellite genotyping of the extracted DNA using multiplex PCR. We amplified 8 microsatellite loci: TuT1, TuT2, TuT3, TuT4, TTT1, Bg12, Bg16 and Bg18 (tetranucleotide repeats; Segelbacher et al. 2000; Caizergues et al. 2001; Piertney & Höglund 2001). Afterwards, unique genotypes were molecularly sexed. Namely, we amplified 2 sex-specific markers: PU and P8mod (Pérez et al. 2011). Molecular sexing is based on the detection of the chromosome-specific difference in intron size in the CHD gene. We tested the reliability of molecular sexing using black grouse and capercaillie individuals of known sex from breeding facilities. We amplified two multiplex regions from the microsatellite loci, as described in Rutkowski et al. (2017b), whereas sex markers were amplified as described in Pérez et al. (2011). We used CEQ 8000 sequencer (BECKMAN COULTER) for the genotyping analyses. In the case of microsatellites we used precautions to avoid genotyping errors following the protocol described in Rutkowski et al. (2017b). Additionally, each sample was genetically sexed in five independent repetitions.

Statistical analysis

The analyses of the microsatellite genotyping were carried out in several stages. First, we identified the samples with the same genotype and, assuming that these samples originated from the same individuals, determined the number of unique genotypes in the studied population. Then, for all unique genotypes, we determined the sex and calculated the Probability of Identity (P(ID), average probability that two randomly sampled unrelated individuals from the same population will have the same genotype) for each locus within each population, as well as for a combination of nine loci. We conducted the analysis using GenAlEx v. 6.501 (Peakall & Smouse 2012).

RESULTS

We successfully genotyped 193 samples (73% of all samples collected): 104 from black grouse ($\approx 63\%$), and 89 from capercaillie (89%). We obtained 44 unique genotypes for the black grouse and 33 for the capercaillie. The P(ID) for the combination of 8 loci was $2.1 \cdot 10^{-7}$ for the black grouse and 2×10^{-6} for the capercaillie

In birds, males are homogametic (sex chromosomes ZZ) and females heterogametic (ZW), thus we classified an individual as male when only a single fragment was amplified in the PCR

reaction (exhibited as a single 'peak' in chromatogram) or as a female when two fragments were amplified (exhibited as the two 'peaks'). We found a sex-ratio of 1:15 for the capercaillie (2 females, 31 males) and a sex-ratio of 1:6 for the black grouse (6 females, 38 males). Multiple repetitions of samples indicated low sexing error. Combination of results from different samples of single individual, revealed by microsatellite genotyping, and repetition of genetic sexing for the particular unique genotype indicated sexing error in less than 10% of cases.

DISCUSSION

The results show a clear deviation from an equal sex ratio with bias towards males in both species, particularly in the capercaillie. There are three possible reasons for such significant differences in the identified number of individuals of both sexes: disturbance in the population demography associated with declining numbers; collection of non-invasive material mainly from cocks due to a varied activity of individuals of both sexes; technical problems resulting from the poor quality of genetic material obtained from non-invasive samples.

Many species of grouse (Galliformes) including the capercaillie and the black grouse, are threatened with extinction, mainly due to the progressive fragmentation of the forest habitat (Caizergues et al. 2003, Brickle et al. 2008, Segelbacher et al. 2008). Among grouse, the sex ratio depends on many factors, and a biased ratio towards one of the sexes is often reported. The differences in the number of individuals of one sex might occur at the level of the breeding cohort and of the entire population (Hörnfeldt et al. 2001, Donald 2007, Kidwai et al. 2011, Wilkinson et al. 2018). In poorer habitats, birds with sexual dimorphism, where males are larger than females, produce more female offspring due to higher mortality of male offspring, which usually need more food to grow faster (Lindén 1981, Moss & Oswald 1985, Moss et al. 2008). In good quality habitats, the sex ratio is usually equal (Ellison & Magnani 1985, Marti et al. 2016). Differences in sex ratios can also be caused by a difference in the predation pressure. Females often lead a secretive lifestyle and have mimetic colorations, whereas the more active and larger males are more vulnerable to attacks, especially during the mating season. Conversely, brooding females are more vulnerable to attacks, e.g. by foxes, as their ability to escape is limited (Clutton-Brock 1986, Clutton-Brock & Vincent 1991). At least in the case of the capercaillie, evidence suggests a system where females are more intensively eliminated from the population. In studies on the reintroduced population in the Lower Silesian Forest, higher survival rates of cocks released into a new environment were observed (Merta et al. 2013). The predominance of males in the population often occurs in small and declining populations is also observed in other birds, e.g. in the willow warbler (*Phylloscopus trochilus*) (Morrison et al. 2016). In the case of game birds, including grouses, hunting is an additional factor that might affect the sex ratio in a population (Zbinden et al. 2018).

The high number of identified males may also be related to the timing of collection of the biological material. The non-invasive samples were collected from February to May, during the snow cover period, when tracks and droppings are easier to spot. This period also corresponds to the mating season of the capercaillie and the black grouse, thus finding male traces in the vicinity of lekking sites is a much easier task. In this period males are very active (Storch 1995, Lindström et al. 1998), whereas hens lead a secretive lifestyle and are more prone to intra-population migration (Storch & Segelbacher 2000, Corrales & Höglund 2012, Rutkowski et al. 2013). Females on the other hand, visit small lek areas less frequently. With the decline in the number and fragmentation of the population, single cocks appear in lek areas. This results in a decrease in the activity of females, and thus they do not leave "traces" in the lek (Alatalo et al. 1992). This regularity is also observed in other groups of birds (Durães et al. 2009). Moreover, for other lekking species (e.g. manakins) it was shown that females only stay in the lek for short

periods, visit numerous lekking sites, have larger home ranges and are thus significantly less bound to the lekking sites as compared to males. Males rarely leave lekking sites, usually only very briefly to feed. It may intensify the effect of uneven probability of sex detection, as the biological traces of the presence of females are dispersed (Théry 1992).

Another reason for such a visible deviation in the sex ratio may be errors resulting from the use of non-invasive samples, such as droppings, as a source of genetic material for sex identification (Bonin et al. 2004, Johnson & Haydon 2007). Apart from the possibility of False Alleles (FA) appearing as the results of samples' contamination, a serious technical problem is also Allelic Drop-out (ADO), i.e. the lack of amplification of one of the microsatellite alleles, especially the longer one (Taberlet et al. 1999, Broquet & Petit 2004). This might result in the heterozygous genotypes to actually be identified as homozygous. The identification method we used was based on identifying homo- and heterozygous individuals, with heterozygosity interpreted as evidence that the focal individual is a female, meaning that ADO could have caused an underestimation of real number of females. In other instances this methods did not cause such biases (the capercaillie non-invasive analysis in the Gorce National Park, unpublished data) and although we employed several measure to prevent identification errors (e.g. multitube strategy) we cannot rule out that some of the genotypes were mistakenly identified as males.

In summary, despite intensive sampling, the observed biased sex ratio may differ from the actual value as sample collection method might have resulted in a higher number of identified cocks. Laboratory errors, even if it occurs, were insignificant due to the procedures used and the scale of obtaining biological material (over 260 samples from both species in total). This leads to the assumption that in the population of the capercaillie and black grouse in the Tatra National Park there is some deviation from the equal sex ratio, biased towards males.

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STRESZCZENIE

[Molekularna identyfikacja płci wykonana na podstawie nieinwazyjnych prób sugeruje zaburzoną proporcję płci w populacji głuszca i cietrzewia w Tatrzańskim Parku Narodowym]

Proporcja płci (stosunek liczby samców do liczby samic) jest znaczącym czynnikiem wpływającym na prawdopodobieństwo przetrwania populacji. Zaburzona proporcja płci może przyczyniać się niekorzystnych procesów demograficznych, szczególnie w przypadku gatunków zagrożonych. Analiza DNA pozyskiwanego z prób nieinwazyjnych jest efektywnym sposobem monitorowania zwierząt rzadkich bądź trudnych w obserwacji. Taką metodę wykorzystano w celu określenia proporcji płci w populacji głuszca (*Tetrao urogallus*) i cietrzewia (*Lyrurus tetrix*) w Tatrzańskim Parku Narodowym. Zebrano ponad 250 prób (gównie odchodów), a następnie przeprowadzono identyfikację osobniczą (genotypowanie mikrosatelitarne) i określono płeć (różnice wielkości intronów genu CHD, sprzężonego z płcią) dla każdego unikatowego genotypu. Analizy genetyczne wykazały, że większość zebranego materiału pochodziła od samców. Odchylenie od równej proporcji płci było bardzo wyraźne. To sugeruje, że w badanej populacji głuszca i cietrzewia jest o wiele więcej kogutów niż kur. Jednakże wyniki należy interpretować ostrożnie, ponieważ na stwierdzone odchylenie wpływ mógł mieć także sposób pozyskiwania materiału w terenie – koguty są o wiele aktywniejsze, w związku z czym o wiele łatwiej trafić na ich tropy oraz ślady biologiczne. Uzyskane wyniki wskazują na potrzebę dalszego monitorowania proporcji płci tych gatunków w Tatrzańskim Parku Narodowym, w oparciu zarówno o metody genetyczne jak i obserwacje.