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SHORT NOTES

Sexing Red-necked Grebes *Podiceps grisegena* by molecular techniques and morphology

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Abstract. Sexual size dimorphism was analysed in the Red- necked Grebe in southeast Poland. A DNA-based procedure was utilised to sex individuals and to assess the accuracy of morphological criteria for the sex identification of adult breeding birds: discriminant analysis on the sample level and within-pair comparisons. Males were significantly larger than females in all body measurements used in the discriminant function selection process. Owing to considerable overlap in measurements, however, the sexes cannot be accurately separated by biometrics at the population scale. Sexual dimorphism was most pronounced in bill length measured from the corner of the gape to the tip, but only 79% of individuals were correctly identified on the basis of this parameter alone. When two variables, bill length and wing length, were combined, the discriminant function was of similar efficiency (80%) in determining the sex. The accuracy level of sexing may be improved by comparing mates within pairs: combined comparisons of bill length and body mass were as accurate as the genetic technique, but sex assignment was restricted to 76% of the measured pairs.

Key words: Red-necked Grebe, *Podiceps grisegena*, sexual size dimorphism, discriminant function analysis, molecular sexing, within-pair comparisons

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Sexing individuals, a prerequisite of many behavioral and ecological studies, is difficult without invasive methods in an array of bird taxa. The recent development of molecular sexing techniques using DNA markers (e.g. Griffiths et al. 1996, Ellegren & Sheldon 1997) provides a reliable non-destructive research tool. Although DNA-based genetic techniques have become common, researchers may prefer simpler methods of sexing by morphology or behavior in order to facilitate the sexing procedure (Mallory & Forbes 2005). Even in species that do not exhibit pronounced sexual dimorphism, slight differences in mensural characters, especially when combined by discriminant analysis, may help to separate the sexes. Also, many birds can be unequivocally sexed from sex-specific behavior, most often by vocalizations, courtship displays or position during copulation (Ainley et al. 1985, Casaux & Baroni 2000). Such options may be preferable when obtaining tissue samples necessary for DNA extraction is difficult or causes serious disturbance to the birds.

Problems with sex identification by morphometry and plumage are particularly true for many grebe species (family Podicipedidae). Also, in most species it is difficult to point out any behavior unique to one sex (Storer 1969, Fjeldså 1973). Progress in grebe research has been additionally hampered by lack of time-efficient, low-intrusion capture techniques. In consequence, few field studies on grebe morphometrics generally refer to birds that died on migration or wintering grounds, where unknown proportions of individuals originating from different populations may contribute to the samples (Fjeldså 1973, Piersma

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1988, Jehl et al. 1998). Due to geographical variation in morphometric measurements, applicability of discriminant functions derived from different samples to distinguish the sex at the scale of local populations may be limited. Conversely, discriminant functions calculated for one breeding locality often apply only to the populations from which they were derived, especially when geographic variation involves shape, which could influence the weighting of characters in the discriminant formula (van Franeker & ter Braak 1993). The requirement to calculate discriminate functions separately for local populations may be omitted by comparing mates within pairs, the use of this method also highly improves the sexing accuracy (Fletcher & Hamer 2003).

The aim of our study was to evaluate morphological criteria for sex determination of the Eurasian subspecies of the Red-necked Grebe *Podiceps grisegena grisegena* and to verify their validity with a DNA-based sexing technique. Sexual size dimorphism in this subspecies appears to be less pronounced than in its North American counterpart, the substantially larger, heavier and longer-billed *P. g. holboellii* (Bocheński 1994, Stout & Nuechterlein 1999).

The study was conducted in a Red-necked Grebes population nesting on fish ponds and small artificial water reservoirs in eastern Poland (50°55′–51°27′N, 21°58′–22°26′E) between 1996 and 2005. Birds were trapped from May through July in underwater nets (Ferguson 1980, Breault & Cheng 1990) or by night-lighting using two-million candlepower spotlights. Captured birds were marked with individual steel and color band combinations.

Genetic sex determination. DNA was isolated from blood collected into EDTA buffer (150 mM NaCl, 15 mM trisodium citrate, 10 mM EDTA pH 7.0). We collected 50–200 µl of blood from the brachial vein of each bird shortly after capture. DNA was extracted from red blood cells using QIAamp DNA Mini extraction kit (QIAGEN) following the manufacturer's protocol. We used protocols described by Kahn et al. (1998) to amplify the intron that intervenes between CHD helicase and DNA-binding regions and is common to both the Z — linked and W — linked CHD genes located on the avian sex chromosomes (Ellegren & Sheldon 1997).

The PCR product was analysed by electrophoresis following Sambrook et al. (1989) using a 3% agarose gel in 1x TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) with 1 µg/ml ethidium bromide and visualized under UV light to reveal the presence of a single or a double band representing male or female, respectively.

As the molecular methods are not universal (e.g. Kahn et al. 1998, Dawson et al. 2001), the technique was verified using samples from 20 birds (10 adults and 10 chicks) found dead in the study area and sexed by dissection and examination of the gonads. The sexes inferred from band patterns appearing on agarose gels agreed with those determined in 20 internally sexed birds. 22 birds were sexed twice from various blood samples (12 of them from samples taken in consecutive years), and the sex designation did not differ. The results were also substantiated by comparing the assigned sex in 17 pairs, where DNA was analysed from both breeding partners. In all cases, male-female pairs were inferred.

Biometrics. Morphometric measurements were made on a total of 76 adult breeding grebes (39 males, 37 females) sexed by DNA. As measurements of some dimensions can be affected by post-mortem changes (Fjeldså 1980, Piersma 1988), we used only living birds and 6 freshly dead individuals, found at the nest.

Standard measurements included body weight, long bill length (gape length from the distal tip of bill to the corner of the mouth), culmen length ("exposed culmen"from the distal tip to the tip of the forehead feathering at the proximal base of the bill), wing length (flattened wing measured from the wrist to the tip of the longest primary) and tarsus length (from middle of mid-tarsal joint to distal end of tarsometatarsus). Dead birds and two live-captured individuals were not weighed. Length of tarsus was taken only over 5 years of study ($n = 47$) and, as the lengths did not differ significantly between the sexes ($t = 1.867$, $df = 45$, $p = 0.068$), this character was dropped from further analyses.

All measurements were taken to the nearest 0.1 mm with Vernier calipers, except wing length that was measured to the nearest 1 mm with a flat ruler. Body masses were taken to the nearest 1 g.

Analyses were based on unique estimates (averages were used for individuals captured on more than one occasion) for each single bird. The Kolmogorov-Smirnov test was applied to test whether the data conformed to normal distribution. The measurements were subjected to stepwise discriminant analysis to select the combination of characteristics most distinct between sexes. Variables were retained in the stepwise model at

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the $p = 0.05$ significance level. Correlation coefficients between characteristics used for the final discriminant function were less than 0.55. The variance-covariance matrices were not significantly different (Box's $M = 9.29$, $p = 0.569$). The discriminatory ability of each variable was evaluated with Wilks' Lambda statistic, which provides the value of the inverse power of discrimination. For each sex and for the whole sample, we report the percentage of individuals correctly identified.

As measurement of body mass may be unreliable because of fluctuations in body condition throughout the breeding cycle (Moreno 1989, Pugesek & Diem 1990), or in some circumstances body mass may be not available or misleading (e.g. carcasses, undernourished birds), its use in discriminant function is usually not recommended (Piersma 1988). Hence, we calculated two functions, one that included and one that excluded body mass as a parameter.

Red-necked Grebe males were significantly larger than females in all body measurements, but there was a considerable overlap between all dimensions measured. Weight was the most variable measurement within sexes (Table 1).

When body mass was not considered, only long bill length (selected firstly) and wing length (selected secondly) contributed significantly to discriminating between males and females (Wilks' Lambda = 0.582, $F_{2,72} = 25.86$, $p < 0.0001$). The following discriminant function was developed to distinguish between male and female grebes:

$$D_1 = 0.315(\text{long bill length}) + 0.067(\text{wing length}) - 28.068$$

Table 1. Body measurements and weight (means ± SE) obtained from breeding Red-necked Grebes caught in eastern Poland 1996–2005 and used in the discriminant functions. Ranges in parentheses, CV — coefficients of variations, N — sample sizes. All t values for comparison of sexes were significant ($p < 0.001$).

	Males			Females		
Variable	$\bar{x}$ ± SE	CV	N	$\bar{x}$ ± SE	CV	N
Weight (g)	820.3 ± 12.3 (635–970)	9.1	37	739.2 ± 11.0 (620–847)	8.7	31
Culmen (mm)	40.8 ± 0.4 (36.7–44.6)	5.4	39	38.2 ± 0.5 (33.8–48.3)	7.4	37
Long bill (mm)	53.8 ± 0.4 (47.5–57.5)	4.3	39	49.9 ± 0.5 (42.3–55.7)	5.9	37
Wing (mm)	177.1 ± 1.0 (163–188)	3.5	39	171.1 ± 1.0 (159–182)	3.5	37

where D is the discriminant score. Birds with scores greater than 0 were classified as male and those with lower scores as female. The discriminant model based on the two measurements correctly predicted 84.6% of the males and 75.0% of the females used for model construction with an overall accuracy of 80.0%.

Accuracy did not increase as a result of including weight (however, note the smaller sample, N = 68). Of the four measurements (long bill length, culmen length, wing length and body mass) only the combined long bill length (selected firstly) and body mass (selected secondly) were different enough for use of sex discrimination (Lambda = 0.596, $F_{2,64} = 21.71$, $p < 0.0001$). The resulting combined function was:

$$D_2 = 0.280(\text{long bill length}) + 0.007(\text{weight}) - 20.236$$

where values of $D_2 > 0$ identified males and values < 0 identified females. This function yielded 76.5% successful allocation of birds in the analysis sample; 83.8% of males were correctly identified, while females had 67.7% correct allocation.

Thus, long bill length considered as the only variable (with 51.9 mm as a point of inter-sex separation) correctly classified 78.9% of the cases (84.6% of males and 73.0% of females) and was the best single factor discriminating between the sexes (Lambda = 0.638, $F_{1,74} = 41.99$, $p < 0.0001$).

Within 17 pairs, where both pair members were caught, in only two pairs did the female have a longer bill than the male (one-tailed sign test, $p = 0.004$); similarly in only two (other) pairs was the female heavier than the male ($p = 0.004$). However, only three pairs were captured on the same occasion and in some pairs the mates were caught in different years. When within a pair, the bird with the larger long bill length or body mass was assumed to be male, the accuracy level increased to 88.2%. Other morphometric measurements, when the larger mate was classified as male, did not differ significantly between sexes (culmen length: $p = 0.052$; wing length: $p = 0.210$). When there was agreement between the measurements of long bill length and body mass, sex assignment was at the same level of accuracy as that obtained from genetics, but the fraction of sexed mates was restricted to 13 out of 17 pairs (76.5%).

Of the characters measured, long bill length was the best single predictor of sex. Adding wing

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length to the model only slightly increased its discriminatory power. However, neither single bill length measurements nor the above combinations of morphometric characteristics provided a complete separation of the sexes. Although males averaged larger than females in all measurements used in our study, the zone of overlap was too large for effective application of discriminant functions; the overall classification success of neither model exceeded 80%.

Although our sample of Red-necked Grebe pairs with both mates caught was small, within-pair comparisons appear to considerably increase the accuracy of sex assignment by measurements of bill and body mass when it is assumed that the larger bird is male. Some studies show that mate-based models are superior to sample-level analysis, but often at the cost of obtaining a smaller sample size (Weidinger & van Franeker 1998, Jodice et al. 2000, Fletcher & Hamer 2003). Moreover, their use eliminates the need to calculate site-specific discriminant functions for different alternative populations (Fletcher & Hamer 2003). This may be particularly helpful in the Red-necked Grebe, as birds in northern Europe are larger and longer billed than in the central part of the continent, presumably as a character release/character displacement resulting from allopatry of the Great Crested Grebe *Podiceps cristatus* (e.g. Fjeldså 1983).

The high accuracy of within-pair comparisons offers a practical solution for the common cases when it is difficult to capture the birds, as the sex of breeding grebes might be predicted from visual inspection of the size of the mates. It is by far not a general recommendation, as it requires caution and careful observations, and should be restricted to those pairs which can be easily watched and distinguished by size. However, the development of techniques allowing quantitative estimates of animal body parts from a distance (Butler et al. 1990, Lyon 1994) may facilitate the use of within-pair comparisons in a wide range of field studies.

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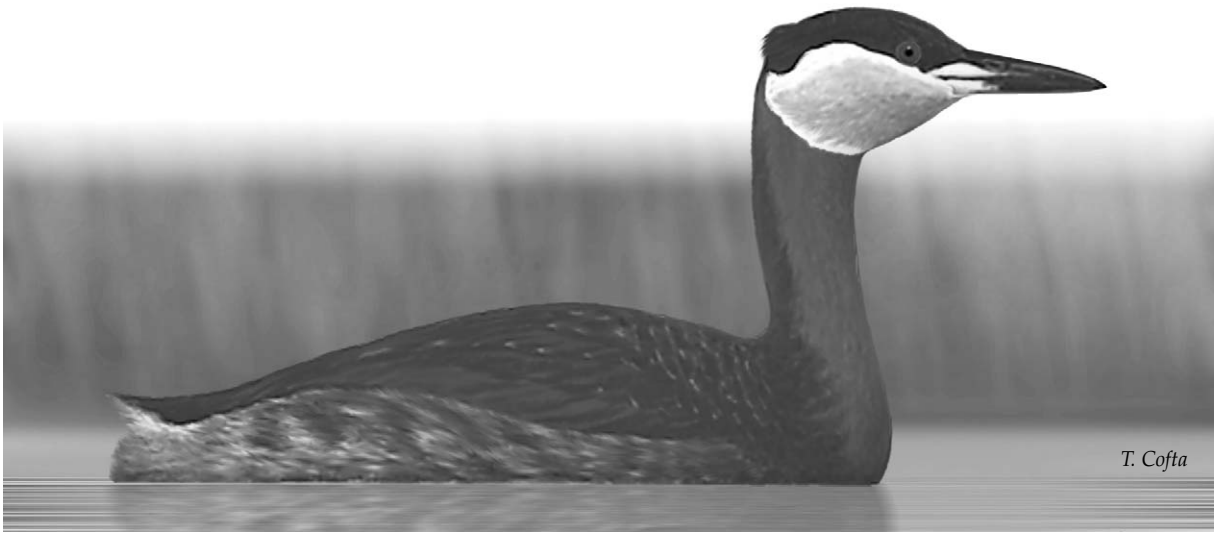
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STRESZCZENIE

[Określanie płci u perkoza rdzawoszyjego za pomocą analizy DNA i pomiarów biometrycznych]

Dymorfizm płciowy wielkości ciała badano w populacji perkoza rdzawoszyjego w płd.-wsch. Polsce. Do ustalenia płci badanych osobników stosowano analizę fragmentów DNA kodujących gen CHD zlokalizowany na chromosomach płciowych. Otrzymane wyniki wykorzystano do weryfikacji dokładności kryteriów morfologicznych oznaczania płci u ptaków lęgowych: analizy dyskryminacyjnej na poziomie próby z populacji i na poziomie porównań osobników w parach. Samce były statystycznie znacząco większe od samic pod względem wszystkich wymiarów ciała użytych w procesie wyboru funkcji dyskryminacyjnej (Tab. 1). Jednak wskutek znacznego podobieństwa zakresów wymiarów u samców i samic, pomiary biometryczne nie pozwalają na dokładne rozróżnienie płci w skali populacji. Dymorfizm płciowy jest najbardziej widoczny w długości dzioba (pomiar wykonywany od czubka dzioba do punktu zejścia się szczęki górnej i żuchwy), ale parametr ten, jeśli użyty oddzielnie, pozwala na prawidłowe ustalenie płci jedynie u 79% badanych osobników. Zastosowanie kombinacji dwóch zmiennych, długości dzioba i długości skrzydła, tylko nieznacznie zwiększyło skuteczność funkcji dyskryminacyjnej (do 80%). Stopień dokładności ustalania płci można podnieść porównując samce z samicami w parach lęgowych: kombinacja porównań długości dzioba i masy ciała okazała się równie wiarygodna jak metoda genetyczna, ale pozwoliła na określenie płci tylko u 76% badanych par. Na atrakcyjność metody porównywania ptaków lęgowych w parach wpływa jej niska inwazyjność oraz możliwość zastosowania do badań populacji innych niż ta, z której pochodzą użyte dane biometryczne.



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