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THE TECHNIQUE OF NONINVASIVE DISTANT SEXING FOR FOUR MONOMORPHIC *DENDROCYGNA* WHISTLING DUCK SPECIES BY THEIR LOUD WHISTLES

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ABSTRACT

Here we present an acoustic approach for reliable sexing in four whistling duck species from the genus *Dendrocygna* and compare it with molecular and cloacal inspection techniques. In the four examined species, the White-faced Whistling Duck *D. viduata*, Fulvous Whistling Duck *D. bicolor*, Cuban Whistling Duck *D. arborea* and Red-billed Whistling Duck *D. autumnalis*, sexes are indistinguishable by appearance. However all the four species show strong sexual differences in the structure of their species-specific loud whistles. For 59 examined birds, an acoustic-based sexing showed 100% accordance to the DNA PCR analysis, while the cloacal inspection showed only 89.8% accuracy. The results demonstrate that acoustic sexing represents a feasible alternative to the two traditional methods as a noninvasive tool for the distant sexing of the four whistling duck species both in captivity and in the wild.

Keywords: sexual dimorphism, call, vocalization, DNA PCR analysis, cloacal inspection, *Dendrocygna*, Anatidae

INTRODUCTION

Birds species without external sexual dimorphism are found in many taxa, such as parrots, cranes, geese, crakes, doves, owls, storks, penguins and goatsuckers (e.g., Clapperton 1983; Cavanagh & Ritchison 1987; Carlson & Trost 1992; Ballintijn & ten Cate 1997; Smith & Jones 1997; Venuto *et al.* 2001; Eda-Fujiwara *et al.*

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2004; Volodin *et al.* 2003, 2005a). Sexing of monomorphic birds is practised in zoos, breeding centres, safari-parks and game facilities for management and censuses or for research purposes in the wild. Four species of the genus *Dendrocygna,* the White-faced Whistling Duck *Dendrocygna viduata* (WF), Fulvous Whistling Duck *D. bicolor* (FU), Cuban Whistling Duck *D. arborea* (CU) and Red-billed Whistling Duck *D. autumnalis* (RB), are commonly kept in zoos and waterfowl collections and three of them (WF, FU and RB) are managed as game species. Visual sexing of any of these species is impossible, excepting observations of copulation (Meanley & Meanley 1958; Bolen & Rylander 1973; Petrie & Rogers 1997a; Volodin *et al.* 2003, 2005a), because the sexes are very similar in plumage and size and both share the parental duties (Johnsgard 1965; Bolen 1970; Clark 1978; Petrie & Rogers 1997a). Some differences in the WF comfort behaviour (higher in females before the egg-lying) or in alertness (higher in males), may not serve as fast and reliable indicators of sex (Petrie & Rogers 1997b). In captivity, DNA PCR analysis (Ellegren 1996; Griffiths *et al.* 1998; Griffiths 2000) and cloacal inspection (Hanson 1949; Purchase 1978) are mainly applied techniques for sex determination in different species of whistling ducks. Both the methods are invasive, as they need capture and handling procedures. At the same time, methods for distant sexing of whistling ducks have not yet been reported anywhere.

Whistling ducks received their name for their characteristic species-specific loud whistles (Johnsgard 1965, 1971). In captivity, the WF, FU, CU and RB produce loud whistles throughout the year upon loss of visual contact with conspecifics or when they hear conspecific loud whistles or their playbacks (Volodin *et al.* 2003, 2005a). Strong sexual differences in the structure of the loud whistles have been reported for all the four species (Volodin *et al.* 2003, 2005a; Volodina *et al.* 2004).

Here we provide a simple algorithm for the reliable acoustic sexing of the WF, FU, CU and RB whistling ducks by call spectrograms and compare the acoustic, molecular and physical examination techniques on the same sample of 59 birds. We took the DNA PCR analysis as a control for the other two methods, as it is reported to have near 100% accuracy (Griffiths *et al.* 1998).

METHODS

Subjects and study site

Our subjects were 59 adult whistling ducks of four species (Table 1): 23 WF (14 males, 9 females), 11 FU (6 males, 5 females), 17 CU (10 males, 7 females) and 8 RB (4 males, 4 females). Nine male and

2 female WF, 3 male FU and 3 male CU were kept in Moscow Zoo (Russia); 5 male and 7 female WF, 3 male and 5 female FU, 7 male and 7 female CU and 4 male and 4 female RB were kept in Tierpark Berlin (Germany). All the birds lived in outdoor enclosures with pools together with other waterfowl. All the birds were marked with individual sets of colour plastic leg rings (Ecotone, Gdansk, Poland).

DNA PCR analysis

We used the DNA PCR sexing technique which has proved useful for many birds (Ellegren 1996; Griffiths *et al.* 1996, 1998; Cerit & Avanus 2007) including Anseriformes (e.g., Quinn *et al.* 1990), and the WF in particular (Jensen *et al.* 2003). DNA was extracted from the dried feathers (3 per bird from a breast region) with DNA-DiatomPrep 200 kit (Isogene laboratory, Moscow, Russia). PCR was performed using primers P8 (5′-CTCCCAAGGATGAGRAAYTG-3′) and P2 (5′-TCTGCATCGCTAAATCCTTT-3′), designed by Griffiths *et al.* (1998). The PCR products were separated by electrophoresis for 80-100 min at 130 V in a 6% denaturing acryl amide gel stained using ethidium bromide and visualized under ultraviolet light. We used the denaturing acryl amide gel because we found that our four object *Dendrocygna* species had only small difference in intron size between the two CHD gene PCR products. In each reaction set, negative as well as positive controls were included to assess the reliability of the method. The PCR was run in a total volume of 20 µl in the original test-tube MasterMix (Isogene laboratory, Moscow, Russia) in amplificator Tercik-TP4-PCR-01 (DNA-Technology, Moscow, Russia). An initial denaturing step of the PCR at 94°C for 90 s was followed by 35 cycles of 94°C for 30 s, 50°C for 60 s and 72°C for 90 s. A final run of 72°C for 6 min completed the program. In electrophoresis, the males showed a single CHD-Z band, while the females showed also a second, distinctive CHD-W band.

Cloacal inspection

All the 59 study birds were sexed by a cloacal examination method (Hanson 1949; Purchase 1978) by an experienced zoo technician. While the male penis is hidden inside a cloaca, it is not visible without a massage for eversion the cloaca. To look for penis presence, the technician held the duck lying on its back over the table with cloaca pointed away from him, extended the tail of the bird out over the edge of the table, bent the tail down over the table edge and applied pressure on the sides of the vent and side towards him. Rotating the finger in a circular motion tended to relax the muscle

TABLE 1

Results of sexing with the cloacal inspection, DNA analysis and acoustic analysis for 59 examined individuals of four species of whistling ducks (WF: White-Faced. FU: Fulvous. CU: Cuban. RB: Red-billed). MZ: Moscow Zoo. TB: Tierpark Berlin. M: male. f: female. N calls: number of the examined calls. Grey cells show cases of misclassification.

Species	Bird	Zoo	Sexing technique			N calls
			Cloacal	DNA	Acoustic	
WF	1	MZ	m	m	m	10
	2	MZ	m	m	m	5
	3	MZ	m	m	m	10
	4	MZ	m	m	m	10
	5	MZ	m	m	m	10
	6	MZ	m	m	m	10
	7	MZ	**f**	**m**	**m**	10
	8	MZ	m	m	m	10
	9	MZ	m	m	m	7
	10	MZ	f	f	f	10
	11	MZ	f	f	f	10
	12	TB	m	m	m	10
	13	TB	**f**	**m**	**m**	4
	14	TB	m	m	m	10
	15	TB	m	m	m	8
	16	TB	m	m	m	10
	17	TB	f	f	f	10
	18	TB	f	f	f	6
	19	TB	f	f	f	10
	20	TB	f	f	f	10
	21	TB	f	f	f	6
	22	TB	f	f	f	10
	23	TB	f	f	f	4
FU	1	MZ	m	m	m	10
	2	MZ	**f**	**m**	**m**	10
	3	MZ	m	m	m	10
	4	TB	m	m	m	10
	5	TB	m	m	m	10
	6	TB	m	m	m	7
	7	TB	f	f	f	10
	8	TB	f	f	f	3
	9	TB	f	f	f	1
	10	TB	f	f	f	10
	11	TB	f	f	f	8

Table 1 continues opposite

Table 1 continued

Species	Bird	Zoo	Sexing technique			N calls
			Cloacal	DNA	Acoustic	
CU	1	MZ	**f**	**m**	**m**	10
	2	MZ	**f**	**m**	**m**	10
	3	MZ	**f**	**m**	**m**	10
	4	TB	m	m	m	1
	5	TB	m	m	m	3
	6	TB	m	m	m	7
	7	TB	m	m	m	3
	8	TB	m	m	m	4
	9	TB	m	m	m	1
	10	TB	m	m	m	2
	11	TB	f	f	f	3
	12	TB	f	f	f	1
	13	TB	f	f	f	1
	14	TB	f	f	f	1
	15	TB	f	f	f	1
	16	TB	f	f	f	3
	17	TB	f	f	f	2
RB	1	TB	m	m	m	9
	2	TB	m	m	m	5
	3	TB	m	m	m	10
	4	TB	m	m	m	1
	5	TB	f	f	f	10
	6	TB	f	f	f	5
	7	TB	f	f	f	2
	8	TB	f	f	f	10

that surrounds the vent. The penis of a male will extend about two to three centimetres.

Acoustic analysis

We recorded the loud whistles from 10 June to 17 September 2001 and from 12 July to 6 September 2004 in Moscow Zoo (17 sessions of 10 to 65 min, 11 hours in total) and from 17 to 24 September 2003 in Tierpark Berlin (16 sessions of 35 to 145 min, 21 hours in total). In Moscow Zoo, the recordings were made in the evening time, after the closure of the zoo for the visitors, while in Tierpark Berlin during the day time. During the recordings, two researchers standing outside the enclosure commented by voice the identity of a caller. The distance to microphone varied from 2 to 20 m. We used a Sony WM-D6C cassette recorder with Sennheiser-845e dynamic cardioid microphone.

For analysis, we randomly selected 10 calls of good quality (not superimposed with wind, noise) per bird, whose identity was confirmed

by both the researchers. For birds with less than 10 recorded calls, we analyzed all of them. In total, we analyzed 200 calls for the WF, 89 calls for the FU, 63 calls for the CU and 52 calls for the RB (Table 1).

All acoustic analyses were made with Avisoft SASLab Pro v. 4.3 (Avisoft Bioacoustics, Berlin, Germany). Calls were digitized with 22.05 kHz sampling frequency and 16-bit precision and high-pass filtered at 0.5 kHz to remove background noise. Spectrograms were created using Hamming window, FFT-length 512 points, frame 50% and overlap 87.5%. These settings provided a bandwidth of 111 Hz, frequency resolution of 43 Hz and time resolution of 2.9 ms.

To predict sex by acoustic analysis, we looked over the loud whistle spectrograms on the screen and selected one key call parameter per species demonstrating obvious sexual dimorphism. For the clearly tonal structure of the WF, FU and CU loud whistles, we selected as the key parameter the maximum fundamental frequency F0 max (Figure 1a-c), whose values we measured from the screen with the reticule cursor. For the RB, we selected as the key parameter the mean duration of a note (NoteDur). To measure this parameter, we measured the duration of the rhythmic end part of each loud whistle from the screen with the standard marker cursor and then divided it into the number of notes within it (Figure 1d). All measurements were exported automatically to Microsoft Excel spreadsheets.

RESULTS

Acoustic sexing

In the WF and FU, the F0 max of the loud whistles was always much lower in males than in females. In contrast, in the CU, the F0 max of males was always higher than in females. In the RB, the mean NoteDur was always longer in males than in females. In all the four species, the values of the measured acoustic parameters did not overlap between sexes (Figure 2) and thus were significant by default, so any statistical analyses were redundant.

Thus, the acoustic approach allows the separation of the loud whistles of males and females by considering only a single parameter per species (Figure 2). In the WF, the call belongs to a male when the F0 max < 4.5 kHz and to a female when the F0 max > 5.3 kHz. In the FU, the call belongs to a male when the F0 max < 2.1 kHz, and to a female when the F0 max > 2.8 kHz. In the CU, the call belongs to a male when the F0 max > 2.6 kHz and to a female when the F0 max < 2.55 kHz. In the RB, the call belongs to a male when the NoteDur > 0.13 s, and to a female, when the NoteDur < 0.12 s.

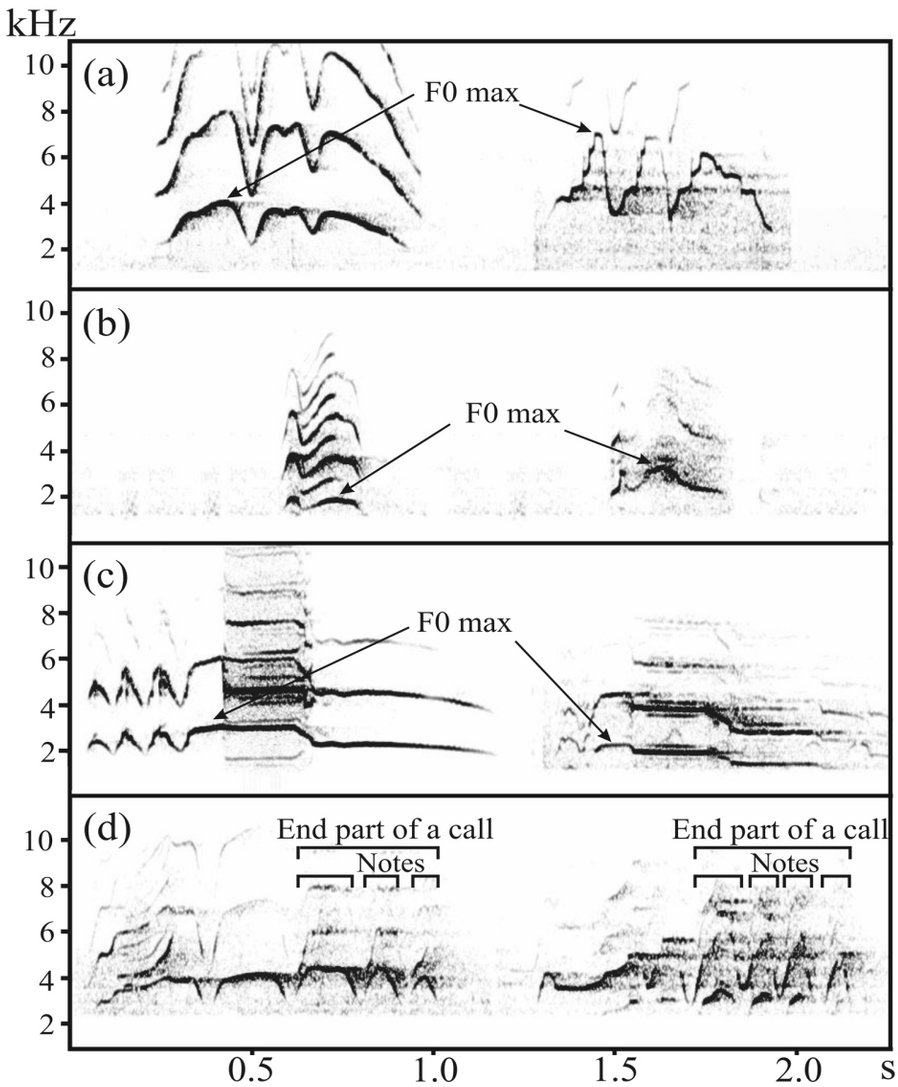

Figure 1. Spectrograms of male (left) and female (right) loud whistles for the four whistling duck species: (a) White-faced; (b) Fulvous; (c) Cuban; (d) Red-billed, illustrating the measurements for the maximum fundamental frequency (F0 max) and for the duration and number of notes within the end part of a call.

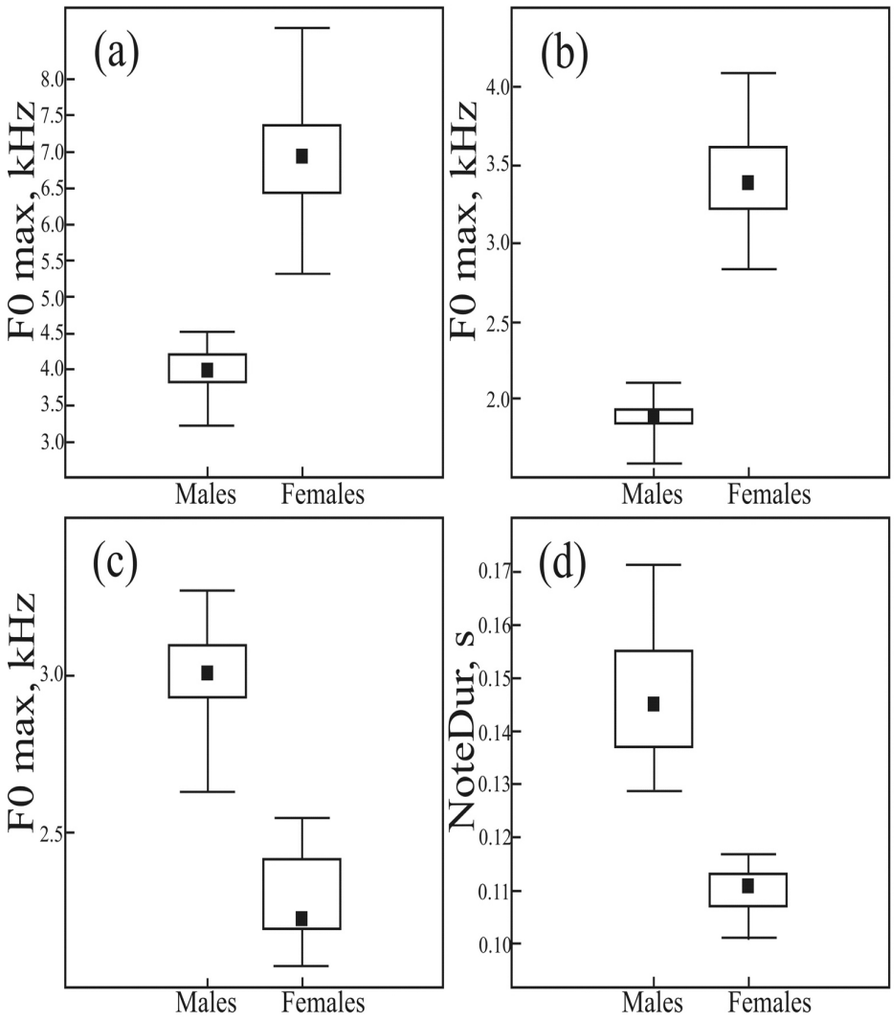

Figure 2. Box plots of values for the loud whistle call parameters, showing the strongest sexual dimorphism in the four examined whistling duck species: (a) White-faced; (b) Fulvous; (c) Cuban; (d) Red-billed. F0 max: maximum fundamental frequency. NoteDur: mean duration of a note within the end part of a call. Points represent medians; boxes (quartiles, whiskers) represent minimum and maximum values.

Concordance between the molecular, acoustic and cloacal sexing

While the results of the molecular and acoustic approaches coincided for all the 59 examined birds, cloacal sexing showed distinctive results for 6 of 59 birds, or only 89.8% accuracy (Table 1). These 6 mistakenly sexed birds belonged to three of the four examined species (WF7,

WF13, FU2, CU1, CU2 and CU3). In all cases of misclassification, males were determined as females (Table 1).

DISCUSSION

In this study, the molecular and acoustic analyses showed 100% reliability of sexing, whereas the cloacal inspection method showed 10.2% misclassification. For the acoustic analysis, we showed that the F0max (for the WF, FU) and the NoteDur (for the RB) were the key parameters that, even taken alone, allowed the sexing of adult birds with 100% accuracy. Below we discuss the relative advantages and disadvantages of the three sexing techniques and consider whether our results with whistling ducks may be generalised to other bird species.

The molecular method is very reliable (up to 100%, Griffiths *et al*. 1998), but relatively expensive, needs a specially equipped laboratory, and do not provides immediate results. Also, human errors and incorrectly designed conditions for the PCR analysis can lower its accuracy in practice.

The cloacal inspection is preferential in zoos, because it provides quick results and does not need special equipment. However, it demands high qualification and practical experience (training) of zoo staff with a certain species or a group of species. With whistling ducks, mistakes of sex determination may occur because the eversion of the cloaca is not always successful in males. All the 6 mistakes occurred during this study favoured a larger percentage of females since the presence or absence of a penis was the only criterion used for sex identification (Table 1). Hence, a penis could be present but overlooked and the bird mistakenly judged as a female. To increase reliability of sexing, more force applied to birds increases the risk of trauma (Turner 1953). This is also the reason why the cloacal sexing is appropriate mostly for adult birds. Although some authors point the possibility of cloacal sexing in young geese and ducks (e.g., Hanson 1949; Bolen 1970), the risk of permanently damaging the developing genitalia of the young birds as a result of this necessarily rather rough handling also must be considered (Turner 1953).

In contrast to DNA sexing, that is basically universal for most birds, the cloacal (vent) sexing technique is not uniform for different bird groups. For example, the cloacal eversion approach used for whistling ducks and other Anseriformes (Hanson 1949; Purchase 1978) is not applicable in some other bird orders. For example, in Columbiformes the cloaca of males typically possesses two conical papillae, one on each side, 1-3 mm in size, that represent the termination of the *vasa deferentia*. The cloaca of females have no papillae and may be identified by the oviduct opening on the

left side, often whitish in colour (Miller & Wagner 1955; Swanson & Rappole 1992). For many Procellariformes, Sphenisciformes and some Gruiformes, the cloacal inspection technique is based on the measurements of the cloaca, which greatly enlarges at egg-laying (Boersma & Davies 1987; Copestake *et al.* 1988). The application of the last technique is limited to a restricted period during and shortly after egg-laying. Also, non-breeding birds cannot be sexed reliably with this technique because non-breeding females have vents similar in size to males (Boersma & Davies 1987).

These differences in the cloacal techniques details may be responsible for the differences in the scores of accuracy reported for this method in application to different bird species. For Columbiformes, the reported level of accuracy is about 90% (Miller & Wagner 1955; Swanson & Rappole 1992), 93-96% for Procellariformes (Boersma & Davies 1987; O'Dwyer *et al.* 2006), 100% for the American Coot *Fulica americana* and 92% for the Magellanic Penguin *Spheniscus magellanicus* (Boersma & Davies 1987).

The acoustic method is non-invasive and allows sexing from a distance that is important in large enclosures, with semi-captive or wild birds. It needs special equipment but is inexpensive as the recordings may be done even with unprofessional equipment and analyzed with freely distributed sound analysis software. Acoustic sexing can provide very quick and reliable results. Just one call per individual was sufficient for 100% reliable sexing of the four examined whistling duck species. The special disadvantage of this method is the necessity to train zoo staff or censuses specialists to record and analyze calls. For the WF and less reliably for the FU sex may be determined by sound recordings, even with the unaided ear, after a short training period with these call patterns; but for the other two species spectrographic analysis of the loud whistles is necessary for sex determination*. However, as a single alternative to capture and manipulations, the acoustical method for sexing the whistling ducks can be recommended both for zoos and for the wild (Volodin *et al.* 2003, 2005a). Another disadvantage is that it is applicable only to adult whistling ducks producing loud whistles. Further data are necessary to determine the age of the appearance of this call type in the vocal repertoire of whistling ducks during the vocal ontogenesis, in order to expand the age limits for the application of this method.

In this study, we used for analysis only recordings of the loud whistles produced spontaneously. However, in zoos keeping only one or a few individuals, or keeping old whistling ducks, it may be difficult to record spontaneous calls for the acoustic analysis, because the birds may be silent. A very effective way to evoke the calling in

*Recordings of the loud whistles for the male and female WF, FU, CU and RBs are freely available from http://www.moscowzoo.ru/get.asp?id=C136)

these birds is to play back the conspecific loud whistles (Volodin *et al*. 2005b). From three old CU males, we could induce 89, 38 and 24 loud whistles per individual within three 10-min-long responses to playbacks. The birds started calling within 10 s from the start of playback and did not show any aggression to each other (Volodin *et al*. 2005b). Our unpublished data showed that the WF and FU also readily respond to playbacks. Our unpublished data on the broadcast of loud whistles to multi-species groups of whistling ducks showed also, that e.g. the WF, FU and CU responded with the loud whistles only to playbacks of conspecific calls, and ignored the loud whistles of other whistling duck species. Therefore, the broadcast the species-specific loud whistles could be very effective to evoke the vocal responses by the loud whistles at least in three species of whistling ducks and allows to record quickly the necessary number of calls for the subsequent bioacoustic analysis.

Acoustic sexing has been developed for many birds without external sexual dimorphism, especially for the numerous Procellariformes which show strong sexual dimorphism in voice. For many Procellariformes, sexing by voice is very successful in the field (e.g., James & Robertson 1985; Brooke 1988; Bretagnolle & Thibault 1995; Genevois & Bretagnolle 1995) and shows very high reliability. For example, both sexes in Leach's Storm-Petrel *Oceanodroma leucorhoa* and Yelkouan Shearwater *Puffinus yelkouan* can be sexed with 100% accuracy by measuring the maximum fundamental frequency alone (Taoka *et al*. 1989; Bourgeous *et al*. 2007). Male and female Whooping Crane *Grus americana* can be sexed with 99% accuracy by guard calls (Carlson & Trost 1992), and male and female Oriental White Stork *Ciconia boyciana* with 100% accuracy by clatter calls (Eda-Fujiwara *et al*. 2004). Collared Dove *Streptopelia decaocto* and Orange-bellied Fruit Dove *Ptilinopus iozonus* can be sexed with up to 100% accuracy by their coo vocalizations (Ballintijn & ten Cate 1997; Baptista & Gaunt 1997). Distant calls of male White-rumped Munia *Lonchura striata* contain only one note, whereas the female calls show rhythmic structure and contain 3.67 notes per call on average. Sexing by ear coincides perfectly with the molecular sexing (Mizuta *et al*. 2003). Strong sexual dimorphism has been reported also for calls of the American Coot and for the crowing calls of the Pukeko *Porphyrio porphyrio* (Gullion 1950; Clapperton 1983). With practice, these differences can be distinguished by ear and used as a quick guide to the sex of birds both in the field and in zoos.

While strong sexual dimorphism in tracheal anatomy has been reported for many Anseriformes (Johnsgard 1961), not many confirmed cases of sexual differences in voices have been reported for the monomorphic Anseriformes. Sexual dimorphism has been found in calls of the Horned Screamer *Anhima cornuta* (Gill *et al*. 1974) and Shelduck *Tadorna tadorna* (Riebesehl-Fedrowitz & Bergmann 1984),

and has been supposed for the Magpie Goose *Anseranas semipalmata* (Johnsgard 1971) and Cape Barren Goose *Cereopsis novaehollandiae* (Baumgarten 1983).

We conclude that acoustic sexing may represent a feasible alternative to the two classical sexing techniques for the four examined species of whistling ducks and has potential as a noninvasive sexing tool for many other monomorphic bird species both in the wild and in zoo management practice. The circus of these species may be expanded with further research.

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