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Sex Determination of Scopoli's Shearwater (*Calonectris diomedea*) Juveniles: A Combined Molecular and Morphometric Approach

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Abstract.—The absence of juvenile sexual dimorphism in birds often impedes sex determination using external morphology. We examined sex ratio variation in Scopoli's Shearwater (*Calonectris diomedea*) fledglings ($n = 135$) on a Strofades island (southern Ionian Sea) between 2007 and 2011. We determined sex by using a polymerase chain reaction-based methodology on blood samples. The molecular technique revealed a slightly male-biased sex ratio (52.6%) during the five consecutive breeding seasons. In addition, morphological analysis using six morphometric variables (bill length, bill width, nalospi, bill-head length, head length, tarsus length) and body weight indicated that males were significantly larger than females for all the tested variables. The combined use of those variables correctly classified 81.2% of known-sex fledglings, and the stepwise discriminant analysis pointed out that bill length was the most discriminating variable followed by bill depth, head length, and body weight. *Received 8 November 2012, accepted 4 February 2013.*

Key words.—*Calonectris diomedea*, discriminant analysis, morphological measurements, Scopoli's Shearwater, sex ratio, Strofades islands.

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Sex determination of fledglings, used to construct the offspring sex ratio, constitutes part of a comprehensive approach to monitor breeding performance in a seabird colony (Griffiths 1992; Bretagnolle and Thibault 1995). Scopoli's Shearwater (*Calonectris diomedea*), recently recognized as a monotypic species (Sangster *et al.* 2012), shows no sex-specific phenotypic plumage traits. This impedes visual sex determination among adults and fledglings (Bretagnolle and Thibault 1995). On the other hand, the species exhibits a large degree of sexual dimorphism among several morphometric variables (Ristow and Wink 1980; Massa and Lo Valvo 1986; Granadeiro 1993). As a consequence, body measurements have been used to identify gender and develop predictive functions for adults and fledglings (Granadeiro 1993; Bretagnolle and Thibault 1995; Lo Valvo 2001).

Usually, discriminant functions that are linear combinations of certain metric characteristics increase the accuracy of sex predictions within a species. In the case of Sco-

poli's Shearwater, however, clinal variation in body size and genetic differences over the species breeding range, as well as several aspects of its biology (e.g., philopatry, nest-site tenacity and mate fidelity) indicate that extrapolations from one colony to another around the Mediterranean Basin could be problematic (Randi *et al.* 1989; Ristow *et al.* 1990; Granadeiro 1993; Rabouam *et al.* 1998). Nevertheless, site-specific sex determination in colonies of Scopoli's Shearwaters is very important to understand their population ecology and manage their populations.

Molecular sexing has been previously applied for the estimation of Shearwater fledgling sex ratio (Genovart *et al.* 2005) and is considered a technique of high accuracy in many avian species (Fridolfsson and Ellegren 1999; Robertson and Gemmel 2006; Bourgeois *et al.* 2007; Thanou *et al.* 2013). Morphometric sexing is quicker and less expensive than sexing with molecular methods, but the effectiveness of discriminant functions needs to be determined before

a choice of the best-suited sexing methodology can be made. In the present study, gender determination of fledglings in an Ionian colony of Scopoli's Shearwaters was implemented by employing a combination of established molecular techniques and discriminant function analysis on morphometric variables. Thus, we evaluated sexual size dimorphism and propose an easy approach for sexing fledglings in this species. In addition, offspring sex ratio was determined during five consecutive breeding seasons in order to evaluate possible ecological correlates within the study area.

METHODS

Study Area

The current work was carried out at a colony of Scopoli's Shearwaters on Stamfani Island ($37^{\circ} 15' N$, $21^{\circ} 00' E$) at the Strofades islands group (south Ionian Sea, Greece; Fig. 1). This island group represents a Natura 2000 site (Natura 2002) and constitutes part of the National Marine Park of Zakynthos in the Ionian Sea. It covers an area of 4 km^2 and is located about 45 km

south of Zakynthos and 49 km west of the Peloponnese (mainland Greece; Fig. 1). Scopoli's Shearwaters nest in five different types of nest sites categorized as: natural shallow cavities under stones, rock cavities among fallen boulders, natural deep cavities under cliff cover, crevices and burrows excavated by the seabirds, and cavities under shrub cover.

Sampling and Data Collection

The study was conducted during five consecutive breeding seasons from 2007 to 2011 during the first and second week of October, when approximately 80% of the chicks had fledged. Scopoli's Shearwater fledglings ($n = 161$) from different nest types of the colony were used. Each individual was banded, and body measurements as well as blood samples were collected. A stratified sampling was followed in order to combine the simplicity of random sampling with the potential increase of survey reliability (Levy and Lemeshow 2008). We recorded body weight (W) and six morphometric variables, namely bill length (BL), bill width (BW) measured at the forward edge of the individual nostril, nares length (NL), bill-head length (BHL), head length (HL) and tarsus length (TL), measured from the distal end of the tarsometatarsus to the base of the last complete scale before the toe diverges, for most fledglings ($n = 139$; 86.3%) (Fig. 2). The measurements were made by the same fieldworker, so as to eliminate possible bias, using a vernier caliper (readable to 0.05 mm) and a 5-kg

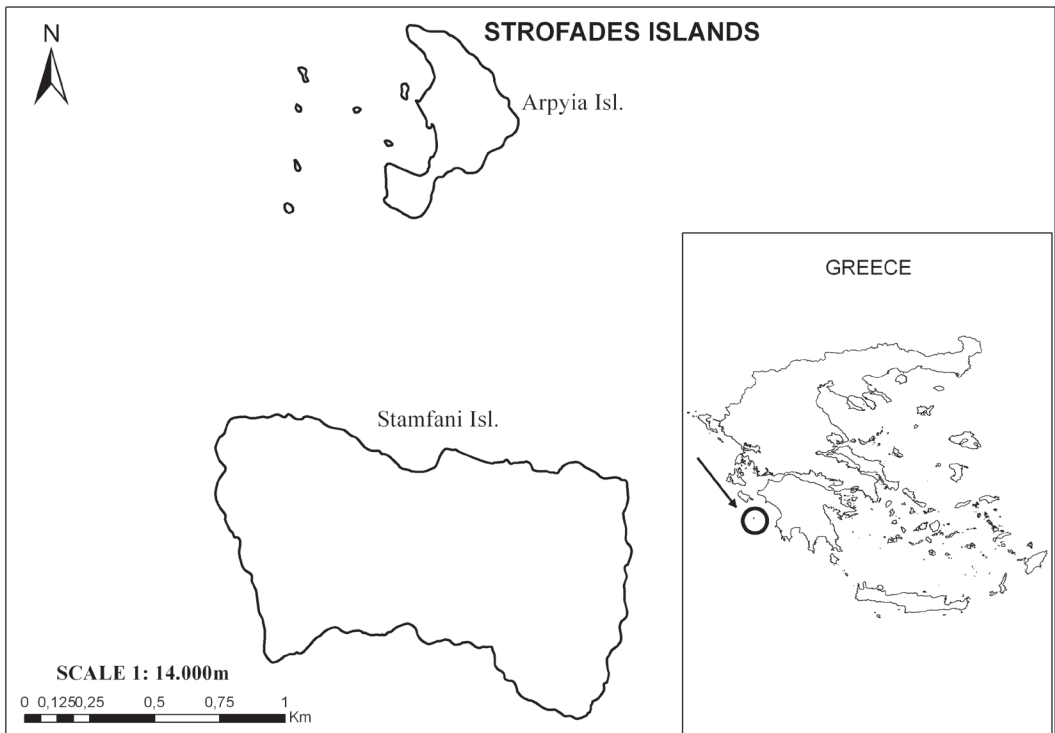


Figure 1. Map of the Strofades island group showing the location of Stamfani Island.

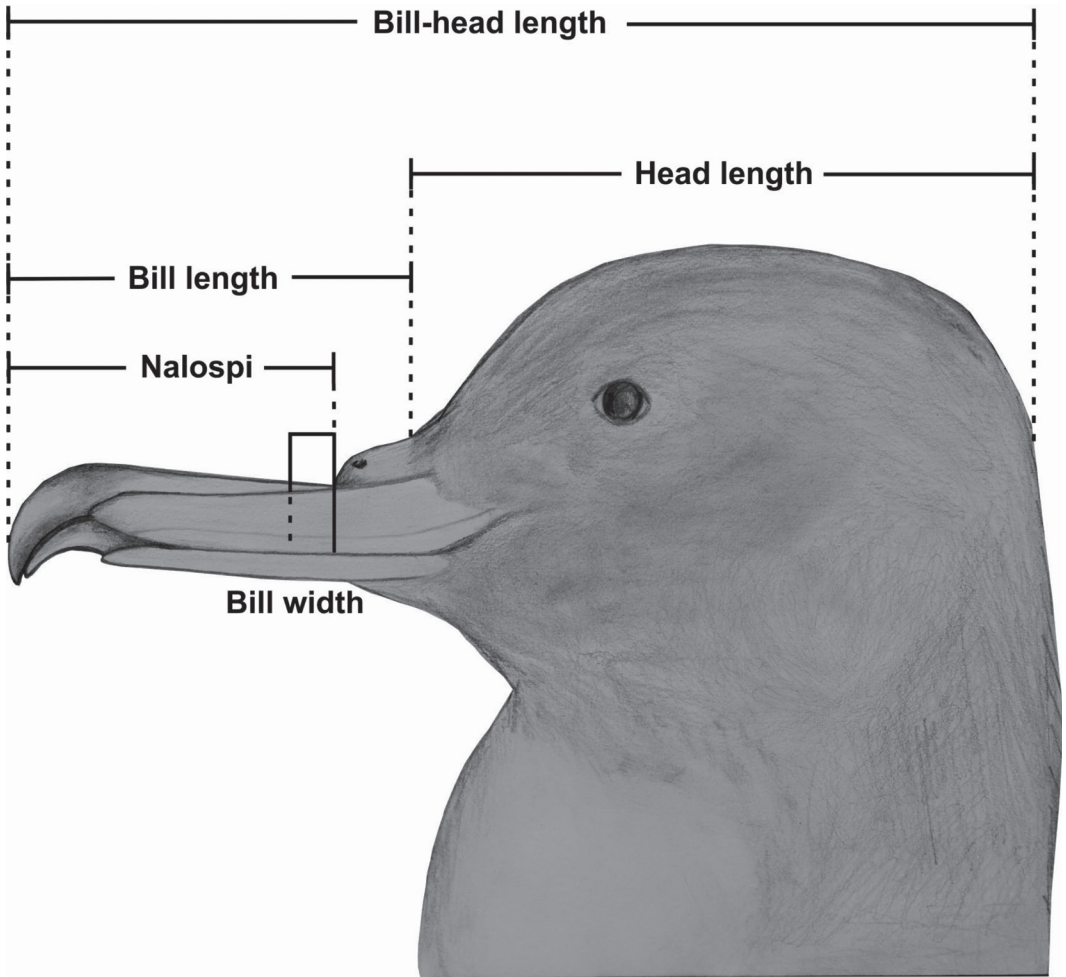


Figure 2. Illustration of head and bill measurements on Scopoli's Shearwater (*Calonectris diomedea*) fledglings.

electronic balance (readable to 5 g). Blood samples of approximately 50 μ l were taken from the leg or the wing vein using non-heparinized capillaries and were stored in a tube with 95-100% alcohol.

Molecular Sexing

To prevent alcohol from interfering with Polymerase Chain Reaction amplification, a small amount (5-10 μ l) from each blood sample was thoroughly washed with TE9 (500 mM Tris-HCl, 20 mM EDTA, 10 mM NaCl, pH = 9.0) and PBS (50 mM KH_2PO_4 , 150 mM NaCl, pH = 7.2) prior to DNA extraction. DNA was extracted following the DNeasy Blood & Tissue Extraction Kit protocol (QIAGEN) and was further processed for the amplification of the avian sex-specific chromodomain helicase DNA binding protein gene (CHD1). The widely applied set of 2550F/2718R primers (Fridolfsson and Ellegren 1999) was used for the amplification of the CHD1 gene, following Genovart *et al.* (2005). Each 10 μ l reaction contained 8 ng/ μ l of genomic DNA, 1.0 mM of each primer and 2.5 U of Taq DNA Polymerase

(Finnzymes) in the manufacturer's buffer, including 1.75 mM MgCl_2 and 0.25 mM of each dNTP. The reaction profile included an initial denaturing step at 94 $^\circ\text{C}$ for 2 min that was followed by 45 cycles of 94 $^\circ\text{C}$ for 30 sec, 48 $^\circ\text{C}$ for 45 sec and 72 $^\circ\text{C}$ for 45 sec, then by a final step at 72 $^\circ\text{C}$ for 10 min.

Polymerase chain reaction products were stained with ethidium bromide under ultraviolet light and run out on 2% agarose gels for approximately 1 hr, in order to provide sufficient resolution to differentiate between the resulting CHD1-Z and W fragments. Fledglings were scored as males when one band was present and as females when two bands were present (Fridolfsson and Ellegren 1999; Genovart *et al.* 2005).

Morphological Analysis

We used one-way ANOVA on each morphometric variable to evaluate sexual differences. Prior to ANOVA, we examined data for assumptions of normality and homogeneity of variance, using Kolmogorov-Smirnov and Levene tests, respectively. Those tests did not reveal

any significant (at 5%) departures from normality or homogeneity. Moreover, we examined possible sexual size-dimorphism. The level of sexual dimorphism (%) in fledglings with known sex for each measurement was calculated as:

$$[(\bar{x}_m - \bar{x}_f) / \bar{x}_f] \times 100,$$

where \bar{x}_m and \bar{x}_f represent the mean values for males and females, respectively.

A stepwise Discriminant Function Analysis (DFA) on morphometric data of molecularly sexed fledglings was used in order to classify the variables by using their discriminatory power according to the Wilks' Lambda statistic (Field 2005). Two variables were not included in DFA: nalospi and bill-head length because they were highly correlated to bill length ($r = 0.75$) and to head length ($r = 0.89$), respectively. A relative discriminant function for sex identification was then developed. The function effectiveness on predicting sex was tested by a cross-validation method. SPSS (2008) was used for the analysis of the data. Only birds for which we had all the measurements ($n = 122$ birds) were considered in stepwise DFA.

RESULTS

Sex Ratio

Of the 161 samples used, 26 could not be amplified. All the remaining samples were unambiguously assigned to the respective sex. Molecular sexing of fledglings revealed a slightly male-biased sex ratio averaging 52.6% over the five examined breeding seasons. When examining sex ratio by year, we found a pronounced bias toward males in only one breeding season (2008), while in 2007, 2009 and 2010 the bias was slight, even slightly in favor of females in 2009 (Fig. 3).

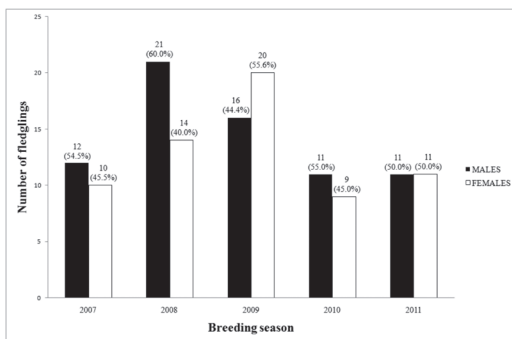


Figure 3. Sex ratio of Scopoli's Shearwater (*Calonectris diomedea*) fledglings on Stamfani Island (2007-2011); n and % per breeding season is indicated on each bar.

None of those differences was significant after a chi-squared test.

Morphometrics

Morphological measurements from the 135 molecularly sexed fledglings of Scopoli's Shearwaters of Stamfani Island revealed sexual dimorphism in seven measurements (Table 1). The level of sexual dimorphism (%) in fledglings was higher in W followed by BW, NL, BL, BHL, HL and TL. According to the ANOVA results, males were significantly larger than females for all the variables tested (Table 1).

A total sample of 122 fledglings that were molecularly sexed and for which all measurements were available was included in the DFA. Pooled within-group correlations between the discriminating morphometric variables and a standardized canonical discriminant function were developed, so as to find the absolute strength of correlation for each tested variable within the function. BL showed the highest discriminant score (0.814) for the sex of Scopoli's Shearwater offspring, followed by BW (0.459), HL (0.400) and W (0.321), whereas TL (0.177) revealed the lowest effect, and the stepwise DFA removed it since it had a limited and insignificant discrimination effect. Therefore, the final discriminant function (D) was based on BL, BW, HL and W:

$$D = 0.003 W + 0.386 BL + 0.431 BW + 0.077 HL - 30.701$$

where values of $D > 0$ identify males. The discriminant function correctly reclassified 82.6% (84.8% males and 80.4% females) of 122 fledglings of known sex (Wilk's lambda = 0.509, $\chi^2 = 79.036$, $P < 0.01$) and cross-validation classified correctly 81.2% of the fledglings (Table 2).

DISCUSSION

The set of 2550F/2718R primers (Fridolfsson and Ellegren 1999) proved to be successful in sex identification of Scopoli's Shearwater fledglings at the study colony.

Table 1. Comparison of body measurements between sexes in Scopoli's Shearwater (*Calonectris diomedea*) fledglings on Stamfani Island (Ionian Sea, eastern Mediterranean), showing ANOVA results (: $P < 0.05$; ***: $P < 0.001$) and percentage of sexual dimorphism for each measurement. Only significantly differing variables are shown.**

Variable	Sex	n	Mean \pm SD	Minimum	Maximum	F	% Sexual
							Dimorphism
Weight (g)	F	64	697.3 \pm 91.27	450	880	7.99 **	9.7
	M	70	764.9 \pm 98.84	490	1000		
	Total	134	732.6 \pm 100.81	450	1000		
Tarsus length (mm)	F	54	52.3 \pm 2.20	46.6	59.3	49.38 ***	2.4
	M	57	53.6 \pm 2.74	41.6	58.6		
	Total	111	53.0 \pm 2.56	41.6	59.3		
Bill length (mm)	F	54	47.7 \pm 2.01	42.0	52.1	27.10 ***	6.2
	M	58	50.7 \pm 1.88	46.7	54.8		
	Total	112	49.3 \pm 2.45	42.0	54.8		
Nalospa (mm)	F	54	36.5 \pm 1.81	32.8	40.2	7.12 **	6.7
	M	58	39.0 \pm 1.77	31.3	42.5		
	Total	112	37.8 \pm 2.16	31.3	42.5		
Bill width (mm)	F	54	12.3 \pm 1.03	10.4	14.7	9.28 **	7.0
	M	57	13.1 \pm 0.93	11.1	16.5		
	Total	111	12.7 \pm 1.07	10.4	16.5		
Bill-Head length (mm)	F	54	100.2 \pm 3.83	94.0	115.0	72.65 ***	6.0
	M	58	106.2 \pm 4.55	99.0	123.0		
	Total	112	103.3 \pm 5.18	94.0	123.0		
Head length (mm)	F	54	52.4 \pm 2.92	46.7	63.3	57.88 ***	5.8
	M	58	55.5 \pm 3.87	45.9	72.2		
	Total	112	54.0 \pm 3.76	45.9	72.2		

The gender of 135 tested fledglings showed a non-significant but slightly imbalanced sex ratio favoring males, and this seemed to slightly fluctuate annually. Bretagnolle and Thibault (1995) found a slightly male-biased sex-ratio on Lavezzi Island after combining data from several years, whereas Genovart *et al.* (2005) found that offspring sex ratios per breeding season in two different Mediter-

anean colonies of Scopoli's Shearwater did not differ from 1:1. It has been hypothesized that a persistent male-biased sex ratio of fledglings could result from predation pressure, since males are more aggressive and can defend themselves from invasive terrestrial mammal species, such as the black rat (*Rattus rattus*), in a more effective way. No significant correlation was found between

Table 2. Testing the accuracy of the discriminant function obtained from the morphometric variables of 122 Scopoli's Shearwater (*Calonectris diomedea*) fledglings on Stamfani Island. In cross-validation, each case was classified by the function derived from all the other cases.

	Sex	Predicted Sex		Total
		Female	Male	
Original	Female (n)	45	11	56
	Male (n)	10	56	66
	Female (%)	80.4	19.6	100
	Male (%)	15.2	84.8	100
Cross-validated	Female (n)	44	12	56
	Male (n)	11	55	66
	Female (%)	78.6	21.4	100
	Male (%)	16.7	83.3	100

breeding success (i.e., number of fledglings per active nest per year) and male-skewed offspring sex ratio during the study period. Furthermore, no significant correlation has been demonstrated between Scopoli's Shearwater breeding success on Stamfani Island and the different levels of black rat activity over the island (D. Petrella, pers. obs.). These results indicate that predation by black rats does not constitute a factor influencing sex ratio of Scopoli's Shearwater fledglings in this colony.

Some of the tested variables were widely used in previous studies and have been successfully applied for sex determination in various seabird species (Lo Valvo 2001; Martínez-Abraín *et al.* 2006; Einoder *et al.* 2008). In our study, males were found to be significantly larger than females for all examined variables, in accordance with previous studies in adult specimens of Procellariiformes (Genovart *et al.* 2003; Copello *et al.* 2006; Bugoni and Furness 2009; Navarro *et al.* 2009).

Mougin *et al.* (1986) demonstrated that the overlap between discriminant scores of Scopoli's Shearwater sexes, calculated as the product of bill length and bill depth in Ristow and Wink (1980), increased with sample size and was stabilized when a sample size of 300 individuals was reached. The discriminant function developed in the current work classified correctly 81.2% of fledglings, a rate of accuracy that is close to the respective scores in previous studies (Bretagnolle and Thibault 1995; Lo Valvo 2001). This result may be attributed to the larger sample used here.

Greater bill length in male Scopoli's Shearwater is in accord with studies in other populations (Ristow and Wink 1980; Triay and Capó 1996) and other petrel species (e.g., Copello *et al.* 2006). The substantial sexual dimorphism of the BL could be linked to differences in the feeding ecology of males and females, such as foraging strategies and dietary preferences (Martínez-Abraín *et al.* 2006; Ballance 2007). More likely, however, the marked sexual dimorphism in bill traits, such as BL, NL and BW, may be attributed to sexual selection related to intraspecific competition for mate and territorial defense

of nesting sites (Bull *et al.* 2005; Bugoni and Furness 2009; Navarro *et al.* 2009).

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