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Genetic and morphological differentiation between the two largest breeding colonies of Audouin's Gull *Larus audouinii*

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We assessed the genetic and morphological differences between the two largest breeding colonies of Audouin's Gull *Larus audouinii*, an endemic seabird species of the Mediterranean region. The two colonies comprise *c.* 75% of the total world population and are 655 km apart. The Ebro Delta colony was formed recently and, after dramatic growth mainly due to high rates of both immigration and reproductive success, is now the largest in the world (more than 60% of the total population). The Chafarinas Islands support an ancient colony with relatively little fluctuation in breeding numbers. The two colonies also differ greatly in environmental conditions, with the Ebro Delta being a higher quality breeding site. Very little movement occurs between the two colonies. We collected morphological data and blood samples from both colonies. Polymorphic microsatellite markers were used to study the genetic differentiation. These showed no significant variation between colonies, nor evidence of a founder effect in the Ebro Delta. Individuals from the Ebro Delta were larger than those from Chafarinas, the difference being greater for males. This probably reflects a stronger male susceptibility to worse environmental conditions during chick growth at the Chafarinas Islands.

In most species, populations are often subdivided by geographical, ecological or behavioural factors. Gene flow between groups homogenizes genetic variation through them. When the rate of gene flow is very low or nil, drift, selection and even mutation may lead to genetic differentiation (Hedrick 1942, Slatkin 1987). In birds, flight, and consequently their dispersal capability, may explain the lack or minor genetic differentiation frequently found between local breeding populations (Ball *et al.* 1988, Burson 1990, Moen 1991, Stangel *et al.* 1991, Ball & Avise 1992, Austin *et al.* 1994, Van Treuren *et al.* 1999, Avise *et al.* 2000). However, despite their mobility, features such as strong philopatry, large geographical scales or historical separations may lead to substantial genetic differentiation between populations of some species. Some examples are several shorebird species (Haig *et al.* 1997), the Lesser Snow Goose *Chen caerulescens* (Quinn 1992), and the Common Murre or Guillemot *Uria aalge* (Friesen *et al.* 1996). Moreover, colonies founded by a small number of individuals can suffer from a loss of genetic variation through the founder effect (Chakraborty & Nei 1976, Hartl & Clark 1989).

Body size in birds has a genetic component (Boag & Van Noordwijk 1987), although environmental factors can also play an important part in determining its variation (James 1983). Many bird species exhibit geographical variation in morphology (Ball *et al.* 1988, Randi *et al.* 1989, Moen 1991, Friesen *et al.* 1996, Wennerberg *et al.* 1999), some of them with no obvious genetic explanation for this variation (Randi *et al.* 1989, Moen 1991). In Audouin's Gull *Larus audouinii* there are no data on possible morphological variation at geographical level, although Ruiz *et al.* (1998) found that environmental factors affected chick growth in a single colony of Audouin's Gull modifying adult size in the long term.

Currently two colonies represent roughly 75% of the world population of the species (Oro 1998): the Ebro Delta colony, formed recently (see below), and

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now the largest in the world (more than 60% of the total population), and the Chafarinas Islands colony, which was formerly the only large colony of this species. The two colonies differ in several ecological features: at the Ebro Delta, competition (inter- and intraspecific) is lower, owing to a high *per capita* availability of suitable nesting sites, and food coming from fishing discards (Oro *et al.* 1996, Oro 1998, Pedrocchi *et al.* 2002). Breeding performance is higher in the Ebro Delta than in the Chafarinas Islands (Ruiz *et al.* 1996, 1998).

The Audouin's Gull has been characterized as relatively philopatric. Oro and Pradel (1999) showed that the probability of breeding at non-natal colonies declines with distance. However, individuals may disperse over large distances (records of natal dispersal up to 750 km) and regularly change their breeding place (Oro & Muntaner 2000, Oro & Ruxton 2001). Demographic studies carried out at the Ebro Delta showed large-scale immigration from other colonies (Oro & Ruxton 2001). However, the estimated transfer rate between the present study colonies is estimated at an average 0.0004% per year, being slightly higher from the Ebro Delta to the Chafarinas Islands (Oro & Pradel 1999, D. Oro unpubl. data).

The purpose of this study was to compare genetic and morphological differences between these two colonies to determine whether they constitute genetically differentiated populations or a single panmictic unit. Taking into account the recent demographic events (formation of a new colony, high migration rates) and life history traits of this gull (high dispersal capabilities, facility to colonize new breeding places), we would not expect genetic differences between colonies. The fact that they differ in environmental conditions leads us to suspect that any morphological differences may be due to the poorer conditions during breeding in the Chafarinas Islands, individuals from this colony being smaller than those from the Ebro Delta.

METHODS

Species and colonies features

The Audouin's Gull is endemic to the Mediterranean region, and is included in the world's lists of species of conservation concern. During the 1970s, it was declared an endangered species since the total number of breeders was estimated at only 800 pairs (Oro 1998). The largest colony was located in the Chafarinas Islands, three rocky and vegetated islands located 4.5 km off the Moroccan coast in the south-western Mediterranean (35°11′N, 2°25′W). This colony, with *c.* 500 breeding pairs in 1966, represented approximately 65% of the total breeding population. In 1981 a new colony was formed by 36 pairs in the Punta de la Banya, a flat and sandy peninsula at the Ebro Delta, in the north-western Mediterranean (40°37′N, 00°35′E). A few years later, the area was protected and the colony grew dramatically to hold 11 725 breeding pairs in 1997, whereas the estimated number in the Chafarinas Islands that year was 2700 breeding pairs. The shortest distance between the two colonies is 655 km (Fig. 1). The western Mediterranean metapopulation of Audouin's Gull was estimated at 17 125 pairs in 1997 (Oro 1998) distributed among several colonies (see Fig. 1).

Sampling

A total of 75 adults (36 in the Chafarinas Islands and 39 in the Ebro Delta) were captured during April and May in 1994 and 1995. Cage traps on incubated nests were used to ensure that only breeding birds were sampled. Several morphological measures were taken: tarsometatarsus length, bill depth at gonys, bill length, nalospi, head and bill length (using a digital calliper ±0.1 mm), wing span (using a tape measure), and body mass (using a 1500-g Pesola spring balance (±10 g)). Blood samples (about 200–500 μL) were taken from the brachial vein using a syringe and collected in a tube with lysis buffer (100 mM Tris HCl, pH 8; 100 mM EDTA, pH 8; 10 mM NaCl; 0.5% SDS) in a 1 : 5 proportion.

Morphological analysis

Since this is a sexually dimorphic species, males being 20% larger than females (Oro 1998), comparisons of measures between colonies were made separately for each sex. Fresh corpses found in the colonies during the study were also measured to increase sample size for the intercolony comparisons. Individuals from Chafarinas were sexed by one of three methods: (a) a previously designed sex-discriminant function (Ruiz *et al.* 1998); (b) gonadal inspection; or (c) polymerase chain reaction (PCR) amplification of DNA (only from blood samples taken from trapped incubating adults during 1994–1995). For genetic sexing we followed Griffiths *et al.* (1998), increasing the annealing temperature to 52 °C (M. Genovart unpubl. data). In total, 80 individuals (36

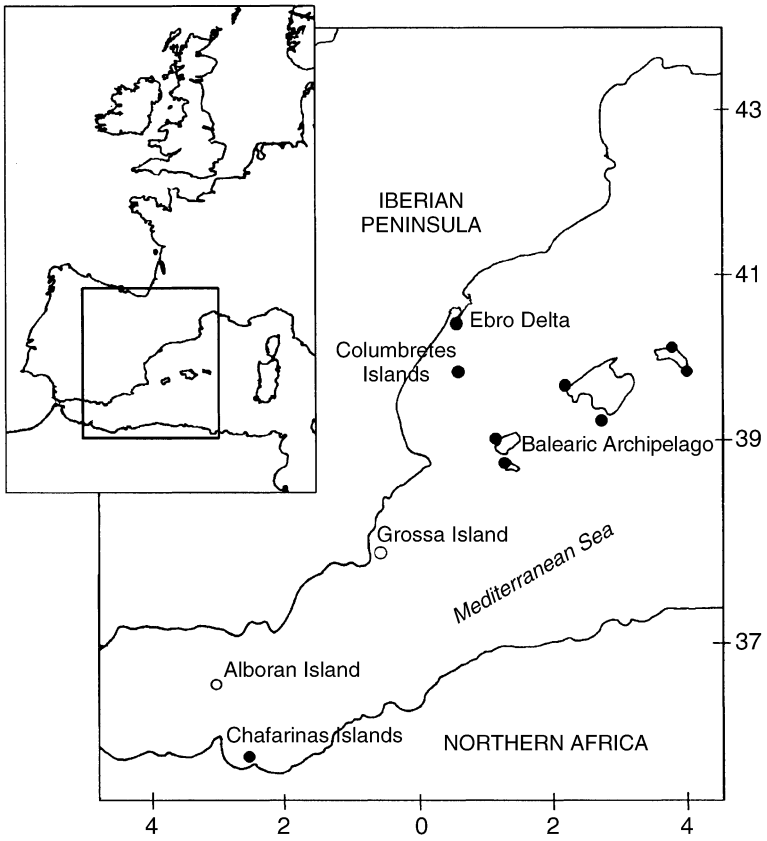

Figure 1. Map of Audouin's Gull breeding colonies from the Western Mediterranean metapopulation. The open dots show the colonies formed in the last decade.

males and 44 females) were sexed by PCR or gonadal inspection at Chafarinas Islands. For the Ebro Delta breeders, we calculated a colony-specific discriminant function using individuals previously sexed, by gonadal inspection of corpses found at the colony or by PCR amplification of DNA from blood samples taken during 1993–1999. The measurements of 89 sexed gulls from the Ebro Delta (46 males and 43 females) were used to obtain several discriminant functions: tarsus–metatarsus length, bill depth at gonys, bill length, nalospi, head and bill length and wing span. Body mass was not used in the analysis. The normal distribution of the measured variables was assessed graphically using normal-plots, and the homogeneity of variances was determined by Levene's test (Levene 1960). Independent variables were not entered at the same time in the analysis. Rather, stepwise discriminant function analysis was performed on all measurements, entering at each step the measurement that added the greatest separation between the two sexes. This was achieved using the Wilks' lambda index (Wilks 1932). The index values range from 0 (mean between groups are different) to 1 (all means are equal). Descriptive statistics and Wilks' lambda between sexes were estimated for the sample to illustrate the sex-discriminating capacity of each variable measured. To assess morphological differences between colonies, comparisons between the body measurements of gulls from the Chafarinas and the Ebro Delta were performed using Student's *t*-test for independent data. All measurements (except body mass of individuals caught at Chafarinas) were made by D.O. either at the laboratory or in the field. Not all the measures were taken from every individual since corpses were sometimes mutilated and their body mass or wing span could not be measured with precision.

Genetic analysis

DNA from the 75 captured adults was extracted with the phenol–chloroform method (Sambrook

Table 1. Microsatellite primers tested in Audouin's Gull. Primers with no information in Variability correspond to those primers that did not amplify microsatellites in this species.

Primers tested	Variability	Species	Reference
D1S500	Monomorph	Humans	Cohen *et al.* (1993)
HrU2	Polymorph	Swallow	Primmer *et al.* (1996)
ADL0176	_	Domestic chicken	http://www.thearkdb.org/browser
GCT0011	_	Domestic chicken	http://www.thearkdb.org/browser
GCT0004	_	Domestic chicken	http://www.thearkdb.org/browser
GCT0013	_	Domestic chicken	http://www.thearkdb.org/browser
GCT0006	_	Domestic chicken	http://www.thearkdb.org/browser
GCT0015	Monomorph	Domestic chicken	http://www.thearkdb.org/browser
GCT0005	Monomorph	Domestic chicken	http://www.thearkdb.org/browser
GCT0003	Monomorph	Domestic chicken	http://www.thearkdb.org/browser
GCT0007	Monomorph	Domestic chicken	http://www.thearkdb.org/browser
GCT0014	Monomorph	Domestic chicken	http://www.thearkdb.org/browser
HG27	Monomorph	Herring Gull	J. Chen unpubl. data
HG25	Monomorph	Herring Gull	J. Chen unpubl. data
HG14	Polymorph	Herring Gull	J. Chen unpubl. data
HG18	Polymorph	Herring Gull	J. Chen unpubl. data
HG1	_	Herring Gull	J. Chen unpubl. data
HG16	_	Herring Gull	J. Chen unpubl. data
HG26	_	Herring Gull	J. Chen unpubl. data

Table 2. Polymorphic microsatellites amplified in Audouin's Gull and PCR amplification conditions in this species.

Locus	Size (bp)	No. of alleles	Temperature (°C)
HrU2	128–140	5	51°
H14	118–126	2	52°
H18	121–125	3	60°

et al. 1989) followed by ethanol precipitation. DNA was dissolved in about 1 mL of TE (1 : 1). Concentrations were evaluated with a spectrophotometer, and samples were adequately rediluted to 25 ng/μL.

To detect genetic differences between colonies we used microsatellite markers. Their small size (generally less than 300 base pairs) allows us to work by PCR amplification, and consequently with small amounts of DNA. Moreover, due to their high mutation rates (Dallas 1992, Ellegren 1995), they are extremely variable, allowing us to find differences in slight departures from panmixia (Bruford & Wayne 1993). Nineteen microsatellite primers designed for other species such as domestic chicken (Schmid *et al.* 2000) (http://www.thearkdb.org/browser), Herring Gull *Larus argentatus* (J. Chen unpubl. data), Barn Swallow *Hirundo rustica* (Primmer *et al.* 1996) and humans were used to amplify Audouin's Gull microsatellites (Table 1). PCR amplifications were performed in 10 or 20 μL reaction volumes using 50 ng of genomic DNA. The programme for the thermal cycler was as follows: 95 °C, 2 min, 30 cycles of denaturing (91 °C, 30 s), annealing (30 s) and extending (71 °C, 1 min). The size of the three primers used in the comparison and their amplification conditions are shown in Table 2. PCR products were electrophoresed on 5% polyacrylamide gels with a known sequence to infer the band size. Primers were radioactively labelled with P^{33} and gels exposed to X-ray film, and developed the day after. We also partially sequenced domains II and III of the control region of mitochondrial DNA in 16 individuals. Polymerase chain reaction amplifications were carried out in 50-μL volumes containing 1 × amplification buffer/IU of Taq DNA polymerase, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP and 0.4 μM of each primer. The amplification primers were L438 (5′-TCACGTGAAATCAGCAACCC-3′) (Wenink *et al.* 1993), and H1248 (5′-CATCTTCAGTGCCATGCTTT-3′). Direct sequencing was performed on an automated sequencer (Amersham Pharmacia Biotech) following the manufacturer's recommended procedures. The sequencing primer was L438. L refers to light and H refers to heavy strands, and the numbers refer to the position of the 3′ end nucleotide of the primer in the White Leghorn Chicken (*Gallus gallus*) mt DNA sequence (Desjardins & Morais 1990).

Statistical treatments for genetic analysis were performed using the randomization approach provided by the Genetix software (Belkhir *et al.* 1996–2001) and Genepop software (Raymond & Rousset 1995).

To determine whether our populations were in Hardy–Weinberg equilibrium (Hardy–Weinberg 1908) we applied the Hardy–Weinberg exact test (Haldane 1954, Guo & Thompson 1992) provided by Genepop (version 1.2, Raymond & Rousset 1995) and a global test across loci and populations was constructed using Fisher's method. We also calculated Wright's *F*-indices (Wright 1951) according to Weir and Cockerham (1984). Wright's F_{st} (Wright 1951) is a measure of the extent to which species show spatial genetic heterogeneity, and is always greater than (or equal to) zero. If all subpopulations are in Hardy–Weinberg equilibrium with the same allele frequencies, then $F_{st} = 0$. Its departure from zero is tested using a global permutation of individuals, conserving genotypes provided by Genetix (Belkhir *et al.* 1996–2001). We used 1000 permutations for all tests. Genetic variation was measured by observed and expected heterozygosity (Nei 1978).

RESULTS

Morphological results

We sexed a total of 82 males and 87 females from both colonies using gonadal inspection or DNA samples (see Table 3). The percentage of individuals from the Ebro Delta colony with correct sex classification using only head and bill length (99% confidence interval [CI]: 115.05–113.13 mm for males, 106.83–104.70 mm for females) was estimated to be between 98% and 100% (Table 4). This allowed us to add 75 males and 75 females for the comparison of body measures between colonies. Discriminant function previously calculated by Ruiz *et al.* (1998) at Chafarinas colony further classified 146 individuals (77 males and 69 females) for the intercolony comparison.

Table 3. Number of Audouin's Gulls sexed at the two study colonies.

		Chafarinas	Ebro Delta	Total
Sexed by gonadal inspection	Males	20	25	45
	Females	24	25	49
Sexed by PCR	Males	16	21	37
	Females	20	18	38
Total	Males	36	46	82
	Females	44	43	87
Total		80	89	169

Significant differences were found in most of the measures when comparing male breeders from both colonies, always being smaller on the Chafarinas Islands (Table 5). For females, only body mass and head and bill length were significantly lower at Chafarinas (Table 5). For both sexes, body mass differed more between the two colonies.

Genetic results

Nine of the cross-species microsatellite amplifications (five designed for domestic chicken and three designed for Herring Gull) did not work in Audouin's Gulls, i.e. they did not amplify any band or they amplified unspecific bands, not corresponding to microsatellites. Eleven microsatellites were finally amplified with some of these primers (Table 1). Two of the Herring Gull primers (H14 and H18)

Table 4. Descriptive statistics of the measurements (in mm, except for body mass in g) used for discriminant analysis for gender determination of gulls from the Ebro Delta according to the sex of Audouin's Gull determined by gonadal inspection or DNA method. Wilks' Lambda statistic shows the sex discriminating power (see text).

	Male					Female					Wilks' lambda
	n	Mean	sd	min.	max.	*n*	Mean	sd	min.	max.	
Body mass (g)	34	669.29	50.79	545.00	755.00	25	563.24	48.51	475.00	655.00	
Tarsometatarsus length	49	59.62	1.83	56.19	62.86	37	55.30	2.05	51.12	58.79	0.269
Bill depth at gonys	49	15.86	0.55	14.89	17.42	40	14.33	0.62	13.42	16.69	0.477
Bill length	49	49.21	1.30	45.71	51.71	40	44.82	1.22	42.18	47.44	0.131
Nalospi	49	25.44	1.02	23.39	27.29	40	23.22	1.13	21.20	26.60	0.116
Head and bill length	27	114.09	1.93	110.74	117.92	23	105.76	1.98	102.10	110.51	0.176
Wing span	20	1378.0	2.97	1300.0	141.00	16	1305.9	2.48	1260.0	1340.0	0.564

Table 5. Body measures of male and female Audouin's Gulls measured at the Ebro Delta and the Chafarinas Islands colonies. Results of statistical comparisons of means are also provided. Lengths are in mm (except wing span in cm) and body mass in g.

	Ebro Delta			Chafarinas						
Sex and measurement	*n*	Mean	sd	*n*	Mean	sd	*t*-value	*df*	*P*	% change
Males										
Body mass	64	659.47	42.84	72	570.21	53.41	10.66	134	< 0.0001	−13.53
Tarsometatarsus length	110	59.74	1.71	112	58.98	2.05	3.04	220	0.003	−1.29
Bill depth at gonys	118	16.16	0.83	97	15.96	0.56	2.05	213	0.041	−1.21
Bill length	118	49.10	1.26	96	48.84	1.81	1.21	212	0.228	−0.52
Nalospi	118	25.19	0.91	97	24.95	0.82	2.05	213	0.042	−0.97
Head and bill length	97	115.18	2.27	87	113.72	1.62	4.95	182	< 0.0001	−1.26
Wing span	20	137.80	2.97	26	135.11	2.46	3.37	44	0.002	−1.95
Females										
Body mass	55	553.96	48.06	63	498.67	34.74	7.22	116	< 0.0001	−9.98
Tarsometatarsus length	110	55.91	2.03	110	55.78	1.73	0.52	218	0.604	−0.24
Bill depth at gonys	120	14.41	0.60	107	14.50	0.63	−1.07	225	0.284	0.06
Bill length	120	44.83	1.37	107	44.84	1.33	−0.08	225	0.939	0.03
Nalospi	120	22.98	0.99	105	22.95	0.78	0.24	223	0.813	−0.04
Head and bill length	103	106.25	1.91	97	105.73	1.65	2.06	198	0.041	−0.49
Wing span	18	130.31	2.68	25	129.41	1.92	1.275	41	0.209	−0.69

Table 6. Allele frequencies and observed (H_o) and expected heterozygosity (H_{exp}) in Chafarinas Islands and the Ebro Delta Audouin's Gull colonies.

Locus	Population	Alleles (named by size in base pairs)					H_{exp}	H_o
HrU2		**128**	**132**	**134**	**136**	**140**		
	Ebro Delta	0.013	0.026	0.307	0.013	0.641	0.4934	0.5385
	Chafarinas	0.043	0.000	0.286	0.043	0.628	0.5196	0.5143
H14		**118**	**126**					
	Ebro Delta	0.500	0.500			0.5000	0.3846	
	Chafarinas	0.428	0.571			0.4898	0.2857	
H18		**121**	**123**	**125**				
	Ebro Delta	0.960	0.000	0.039		0.0758	0.0789	
	Chafarinas	0.986	0.014	0.000		0.0282	0.0286	

and the one designed for swallows (HrU2, Primmer *et al.* 1996) proved to be polymorphic in Audouin's Gulls, and only these were used to assess the genetic differences between colonies.

A moderate level of allelic diversity was observed in two of the three loci (H14 and HrU2): 2–5 different alleles per locus (see Table 6). Five alleles were found at the HrU2 locus. This primer previously worked in a great variety of species (Primmer *et al.* 1996), but never showed more than four alleles. One locus (H18) was almost monomorphic, with a predominant allele found in both colonies and two rare alleles found, one in each colony.

No Hardy–Weinberg departures were found with the Hardy–Weinberg exact test (Fisher's global test: Ebro Delta $P = 0.641$, Chafarinas $P = 0.267$). We found no difference in genetic diversity (measured as H_{exp}) (see Table 6), and no significant departures from zero were observed in F_{st} values (see Table 7).

Table 7. Estimates of F_{st} and F_{is} (see Methods section for explanations).

Locus	Delta F_{is}	Chafarinas F_{is}	F_{st}
HrU2	−0.078	0.025	−0.0102
H14	0.243	0.437	−0.0141
H18	−0.028	0	0.0088
Mean	0.075	0.220	−0.011

We sequenced roughly 500 base pairs from domains II and III of the mitochondrial control region in 10 individuals from the Ebro Delta colony and six individuals from the Chafarinas Islands. We found no variation between the colonies.

DISCUSSION

Our genetic results characterize Audouin's Gull in the western Mediterranean as a panmictic population, individuals being able to breed at any suitable site throughout the region (see Fig. 1). As expected, probably due to the little time elapsed since the new colony's formation, we found no evidence of genetic drift at the Ebro Delta. These results agree with demographic studies showing that the observed growth of the Ebro Delta colony cannot be explained without postulating large-scale immigration from elsewhere (Oro & Ruxton 2001). Although immigration from Chafarinas to the Ebro Delta is now at an extremely low rate (Oro & Pradel 1999), it could have been higher when the colony was formed, since the only large colony at that time was the Chafarinas Islands (Oro & Ruxton 2001). Moreover, founding events involving individuals from several sources within metapopulations can suppress genetic variation between colonies (McCauley 1991).

In several other bird species that are also considered to be philopatric, such as the Snow Goose, Brünnich's Guillemot *Uria lomvia* and the Short-tailed Shearwater *Puffinus tenuirostris*, studies have failed to find genetic differentiation at neutral genes among populations (Avise *et al.* 1992, Birt-Friesen *et al.* 1992, Austin *et al.* 1994, Van Treuren *et al.* 1999, but see Friesen *et al.* 1996). These results suggest that: (a) birds are very mobile and even a low frequency of exchange of breeding individuals may override the genetic divergence, and (b) extinction and establishment of colonies are frequent in many seabird species (e.g. Erwin *et al.* 1998, Oro & Muntaner 2000), and could lead to a frequent exchange of individuals and therefore of genes.

It still remains to be seen whether the absence of genetic differentiation in the Audouin's Gull holds for other colonies across the Mediterranean. When dispersal is high in frequency (as is characteristic of Audouin's Gull, see Oro & Martinez-Vilalta 1994, Ruiz *et al.* 1996, Oro & Pradel 1999, Oro & Ruxton 2001), immigrants from distant metapopulations can be involved in the growth of a local population (Hansky 1998). However, since distance between colonies influences the likelihood of dispersal (Oro & Pradel 1999), gene flow between eastern, central and western Mediterranean metapopulations may be sufficiently low to allow genetic differentiation.

Since our results show no genetic differentiation between the two colonies, differences in body size are probably due to environmental factors. This has been found in Atlantic Puffins *Fratercula arctica* (Moen 1991) in which variation in body size was postulated to be the combined effect of ocean temperature and food quality. Environmental variables also seem to play an important role in maintaining a morphological cline in the Cory's Shearwater *Calonectris diomedea* (Randi *et al.* 1989). Differences in breeding conditions previously recorded at our two study colonies (especially in food supply) suggest that adult morphological differences arose during chick growth; conditions being more favourable at the Ebro Delta than at Chafarinas Islands (Ruiz *et al.* 1996, 1998). Food availability during breeding is a common factor determining body size variability in birds (Boag 1983, 1987, Leafloor *et al.* 1998). Cooch *et al.* (1991) also attributed the decline in size of breeding Snow Goose to a long-term reduction in food availability affecting gosling growth. As recorded in our study, differentiation in size and shape among populations are frequently more marked in males (Ross & Baker 1982, Livezey 1986, Rasmussen 1994), also suggesting that they can be affected more by environmental constraints than can females.

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