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Sex identification in Gentoo (*Pygoscelis papua*) and Chinstrap (*Pygoscelis antarctica*) penguins: Can flow cytometry be used as a reliable identification method?

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ABSTRACT

An important scientific question in ecology is how to differentiate males from females that have similar morphology. In penguins, due to their monomorphic plumage, it is difficult to determine gender. So far, most approaches to address this problem have focused on using sex based discriminant functions combined with DNA based tools as a validation method. As discriminant functions can be species and locality specific, in this study we explored the feasibility of using flow cytometry to determine the sex of two species of penguins, *Pygoscelis antarctica* and *Pygoscelis papua*. Our results for sex assessment determined by flow cytometry were compared with those obtained using morphological characters (bill length and depth), and DNA based methods (using the PL/PR primer pair) were used to validate both approaches. For both species, statistically significant differences were observed between males and females, with males presenting on average 2.1% more nuclear DNA than females. Flow cytometry enabled similar or better rates of correct sex assignment (86.4% and 80.0%) than sex discriminant functions (31.8% and 90.0%) for *P. antarctica* and *P. papua*, respectively, and thus may be considered as a promising alternative to the use of morphological data for sex identification. Nevertheless, some individuals with intermediate genome size values were observed, which increased the difficulty of categorically assigning their sex. Therefore, flow cytometry alone cannot be considered in all cases, but if used together with DNA-based methods in targeted samples, it can be used to efficiently estimate the gender of, at least, these two penguin species, with the potential to be used in other species.

1. Introduction

An important question in ecological research is how to differentiate males from females that have similar morphology. Within vertebrates, birds are generally difficult to sex (Catry et al., 2005; Griffiths et al., 1998). Seabirds are particularly interesting as there are numerous species that exhibit very similar morphologies, such as albatrosses, petrels and penguins (Knox, 2007; Tickell, 2000). Currently, there are six different genera of penguins (Family Spheniscidae) living in the world. Penguins of the genus *Pygoscelis* comprise three species, found mostly in the higher latitudes of the sub-Antarctic and the Antarctica (Davis and Renner, 2003). Gentoo Penguins (*Pygoscelis papua*) have the most northerly distribution, occurring on most of the sub-Antarctic islands but extending to the Antarctic Peninsula, while Chinstrap Penguins (*Pygoscelis antarctica*) are found almost exclusively at the Antarctic Peninsula, further in the south and across the Scotia Arc. At the South Shetland Islands the distribution of both species overlap and both species often breed in close proximity (Davis and Renner, 2003). Knowledge about the ecology and population dynamics of penguins in the Antarctic has grown considerably in the last two decades (e.g. Carravieri et al., 2013; Croxall et al., 2002; Dann et al., 1995; Fretwell et al., 2012).

Sex determination in penguins through visual clues is considered to be difficult due to their monomorphic plumage (Polito et al., 2012; Valenzuela-Guerra et al., 2013). Still, penguins exhibit a slight dimorphism of size between sexes, with males usually having larger body, bill and flipper sizes (Davis and Renner, 2003). This has led to the development of discriminant methods based on single or multiple morphological characters (e.g. Amat et al., 1993; Renner et al., 1998; Setiawan

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et al., 2004). In the case of the *Pygoscelis* penguins, discriminant functions have been calculated previously, and further validated using DNA-based molecular methods for sex determinations (Polito et al., 2012; Valenzuela-Guerra et al., 2013). However, as observed for other penguins, a geographic morphological variation is found in *Pygoscelis* penguins, with decreases in size being observed toward southern latitudes. Therefore, discriminant sexing functions are only valid for specific species/sub-species and localities. This is evident from the study of Valenzuela-Guerra et al. (2013), who developed morphological discriminant functions for Gentoo penguins from three localities in the South Shetland Islands and the Antarctic Peninsula, and observed percentages of correct identification ranging from 83.95% to 93.87% after validation with molecular sex identification, as well as morphological divergence between localities.

DNA-based molecular methods, mostly using universal primers as the P2/P8 pair, have been used in the past as a complementary method to determine the sex of penguins, and, as referred above, as a validation method of the morphological discriminant functions (Griffiths et al., 1998; Polito et al., 2012; Valenzuela-Guerra et al., 2013). The P2/P8 primers have been widely used in avian molecular sexing and amplify regions of the *CHD1* gene found in sex chromosomes (Griffiths et al., 1998). More recently, Zhang et al. (2013) developed primers specific for determining the sex of penguin species, the PL/PR primer pair. In penguin species this primer combination can be used to unequivocally determine the gender of individuals from all species tested, whereas it is not possible to determine the sex in one of the species using P2/P8, due to the production of faint PCR bands of similar size of the *CHD1Z* and *CHD1W* genes.

In the beginning of the 1990s, an alternative sexing methodology was proposed by Nakamura et al. (1990), i.e., the use of flow cytometry to estimate the nuclear DNA content of erythrocytes from a small blood sample. This approach is based on the sex-chromosome heteromorphism present in birds, the ZW sex-determination system. Contrarily to the XY sex-determination system found in humans and most other mammals, males are the homogametic sex (ZZ), while females are the heterogametic sex (ZW). As the Z chromosome is larger than the W chromosome, males have a larger DNA content than females, which considering the precision of flow cytometry in distinguishing minute differences in DNA content (e.g. De Vita et al., 1994; Nakamura et al., 1990), would make it theoretically possible to distinguish males and females based on their genome sizes, only. Indeed, flow cytometry was used with success to distinguish the sex of dozens of bird species from a variety of orders (e.g. De Vita et al., 1994; Nakamura et al., 1990; Tiersch and Mumme, 1993). The difference in DNA content between sexes may vary among bird species (e.g. 0.6% in *Tyto alba* to 5.8% in *Neophron pernocterus* subsp. *ginginianus*, De Vita et al., 1994); this difference is the main determinant of the success of this technique for sexing purposes. In comparison with other methods used to determine the sex of birds, flow cytometry is considered to be a rapid, noninvasive and inexpensive approach (considering that a flow cytometer is readily available) that only requires microliter volumes obtainable from a variety of bird tissues, as long as it is possible to extract any type of nucleated cells.

Despite its potential, after publication of a number of studies, only a few laboratories continued to exploit this technique for determining the gender of bird species. Therefore, the main objective of this study was to evaluate the feasibility and accuracy of using flow cytometry to determine the sex of penguins, in particular of Gentoo and Chinstrap penguins. For that, the results of sex assessment were compared with those obtained through the discriminant function of Polito et al. (2012) based on bill measurements. Also, DNA based molecular methods were used in a subset of samples to validate the flow cytometric results. To our knowledge, this is the first study exploring the use of flow cytometry for such purposes in penguins, being of particular importance considering the difficulty of determining gender in this family.

2. Material and methods

2.1. Field sampling

The Gentoo and Chinstrap penguin blood samples for sex determination were collected in December 2011 and January 2012, at Livingston Island (Antarctic Peninsula) at Hannah Point (60 37′ W 62 39′ S) and at Hurd Peninsula (60 25′ W 62 43′ S), respectively. To reduce disturbance at the breeding colony adult penguins were selected randomly and captured while travelling between the colony and the sea. While all the individuals sampled had adult plumage, this method did not allow us to verify their actual age or breeding status. Blood samples were collected with a 25 ga needle and 1 mL syringe from the brachial vein on the underside of the flipper; this vein is located in the brachial groove that can be felt running along the length of the flipper, approx. 1.5 cm from the leading edge. Blood samples were maintained in a −20 °C freezer for later analyses (see below). Penguin capture, sample collection and subsequent release lasted generally no longer than 10 min. Our methods adhered to all recommendations advised by the Scientific Committee for Antarctic Research (SCAR).

2.2. Morphological sexing

Morphometric measurement is the simplest method available. Bill (culmen) length and bill depth (taken through the centre of the nostrils) were measured to an accuracy of 0.1 mm using vernier callipers. All measurements were conducted by the same scientist. Data was used to calculate the sex discrimination functions and the posterior probability for a male individual, following the methods of Polito et al. (2012).

2.3. Flow cytometric sexing

The genome size of 29 individuals of *P. antarctica* and 28 individuals of *P. papua* was estimated using flow cytometry following the method of Tiersch and Mumme (1993), with modifications. Male chicken erythrocytes, with known nuclear DNA content (2C = 2.33 pg DNA; Galbraith et al., 1983), were used as an internal reference standard. This standard was chosen because its genome size was close to, but not overlapping with that of the sample species.

For each measurement, approximately 1–10 μL of blood of the sample species was mixed with 2 μL of the internal reference standard and added to 1 mL of WPB buffer (0.2 M Tris–HCl, 4 mM $MgCl_2 \cdot 6H_2O$, 1% Triton X-100, 2 mM EDTA $Na_2 \cdot 2H_2O$, 86 mM NaCl, 10 mM metabisulfite, 1% PVP-10, pH adjusted to 7.5 and stored at 4 °C; Loureiro et al., 2007). The suspension was then filtered through a 30 μm nylon filter and nuclei were stained with the intercalating DNA dye, propidium iodide (PI) at 50 $\mu g \cdot mL^{-1}$. As PI also intercalates into double-stranded RNA (Doležel et al., 2007), RNase at 50 $\mu g \cdot mL^{-1}$ was also added to eliminate it.

Samples were kept at room temperature to incubate for 5 min and were subsequently analysed in a Partec CyFlow Space flow cytometer (Partec GmbH, Görlitz, Germany) equipped with a 532 nm green solid-state laser, operating at 30 mW. Each day, during the analysis of the first sample, the amplifier system was set to a constant voltage and gain, with the internal reference standard G_1 peak being positioned on channel 200. The following samples were measured using the same instrument settings.

Data from each sample was acquired using FloMax software v2.5 (Partec GmbH, Münster, Germany) in the form of four graphs: relative fluorescence intensity of PI-stained nuclei (FL) histograms (1), FL vs. time cytograms (2), FL vs. side light scatter in logarithmic scale cytograms (3) and FL height vs. FL area cytograms (4). In the last types of cytograms, regions of interest, comprising mostly the isolated nuclei, were created to eliminate undesirable low-channel

signals, doublets and other debris. Whenever possible, at least 5000 nuclei in both the sample and standard G_1 peaks were analysed per sample.

The quality of the analysis was assessed using the same software, through the careful evaluation of peak symmetry and peak coefficient of variation (CV, %). Only when peaks were symmetrical and CV values were below 5% was the sample considered for genome size estimations.

The genome size in pg (2C) of each individual (GS_s) was estimated using the following formula: $GS_s = G_{1s} / G_{1r} \times GS_r$, where G_{1s} and G_{1r} are the mean G_1 fluorescence of sample and reference nuclei, respectively and GS_r refers to the genome size of reference nuclei.

In order to assess the reproducibility of the obtained results, when the quantity of a blood sample was sufficient, three replicates were performed per individual. Also, these measurements were done in three different days to compensate for any influence regarding instrumental drifts, as advised by Doležel et al. (2007).

2.4. Molecular sexing

Molecular sexing was performed on a sub-set of individual adult Gentoo (n = 10) and Chinstrap (n = 22) penguins. DNA extraction was performed using the Wizard Genomic DNA Purification Kit (Promega, USA), according the manufacturer's instructions. Polymerase chain reaction was used to determine the gender of the individuals, using a pair of primers PL (5′-CCC AAG GAT GAT AAA TTG TGC-3′)/PR (5′-CAC TTC CAT TAA AGC TGA TCT GG-3′) to amplify the *CHD1* gene as described by Zhang et al. (2013). PCR reactions were performed on a MJMini Thermal Cycler (BioRad, USA) with the final volume of 50 μl containing 1× PCR buffer, 0.5 μM of each primer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.25 U Taq DNA polymerase and 10–100 ng genomic DNA. All PCR reactions were initiated with an initial denaturation (95 °C/15 min), followed by 36 cycles of denaturation (94 °C/30 s), annealing (55 °C/90 s) and extension (72 °C/60 s), and ended with a final extension (72 °C/10 min). Blood from male and female chickens were used as positive controls.

2.5. Statistical analyses

Differences in genome size between species and sexes were evaluated with a two-way ANOVA. When treatments were significantly different, a Tukey multiple comparison test was used for pairwise comparison (SigmaPlot for Windows Version 12.0, Germany).

3. Results

3.1. Morphological sexing

The results of morphometric analyses, in particular the measurements of bill length and bill depth, are given in Table S1.

Using the discrimination functions of Polito et al. (2012), for Chinstrap penguins, a total of 75.9% (22 out of 29) of the individuals were assigned as females, and 24.1% as males (7 out of 29). In Gentoo penguins, a total of 57.1% were assigned as females (16 out of 28), whereas 42.9% were assigned as females (12 out of 28).

3.2. Flow cytometry sexing

With flow cytometry, it was possible to obtain PI fluorescence histograms of good to excellent quality (Fig. 1), with two distinct G_1 peaks of sample and standard nuclei. Indeed, the mean CV values of sample and standard G_1 peaks were of 3.08% (ranging from 1.71% to 4.89%) and 2.73% (ranging from 1.57% to 4.28%), respectively. Also, regardless of the species, the measurements were reproducible, with the CV of genome size measurements, being always below 2.5% (0.88% on average), which sustains the good reproducibility of the flow cytometric method.

No significant differences in genome size were observed between both species ($F = 0.0885$, $P = 0.767$; Table 1). Genome size values ranged from 2.85 to 3.12 pg/2C, and after comparison with the results of molecular sexing, two genome size classes were established, 2.85 to 2.93 pg/2C assumed as female individuals, and 2.96 to 3.12 pg/2C considered to correspond to male individuals. There were some cases where intermediate values of 2.94 and 2.95 pg/2C were observed. Once again, using the results of molecular sexing it was decided to consider 2.94 pg/2C as the highest genome size value for female individuals, and 2.95 pg/2C as the lowest genome size value for male individuals. Following this gender classification strategy, in *P. papua*, males (2.98 ± 0.04 pg/2C) had on average 2.3% more nuclear DNA than females (2.91 ± 0.03 pg/2C), while this difference was slightly higher in *P. antarctica*, reaching on average 2.6% (males: 2.99 ± 0.04 pg/2C; females: 2.91 ± 0.03 pg/2C; Table 1). These genome size differences were statistically significant at $P < 0.001$ regardless of the species ($F = 49.78$, $P < 0.001$). Also, no statistically significant interaction between sex and species ($F = 0.168$, $P = 0.684$) was observed.

If we consider only the subset of samples where both DNA-based methods and flow cytometry were used, the genome size difference between males and females was lower in both species (1.0% for *P. antarctica* and 1.4% for *P. papua*).

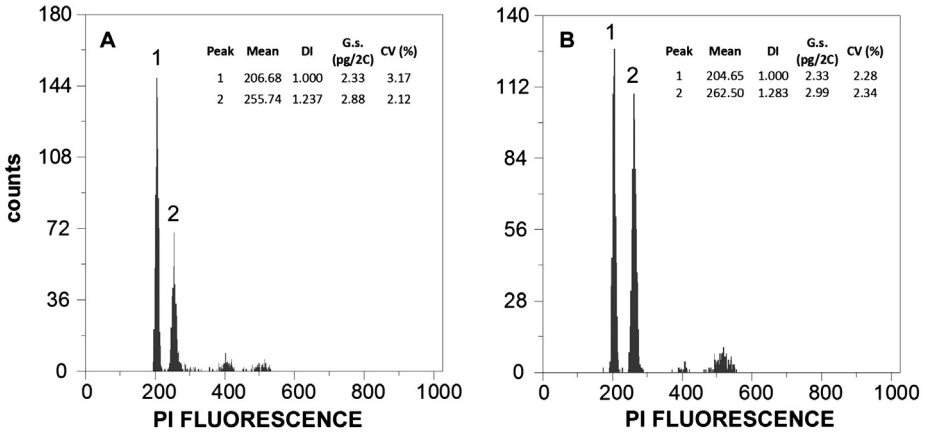

Fig. 1. Histograms of relative nuclear DNA content of domestic chicken red blood cells, as an internal reference standard (peak 1) and *Pygoscelis antarctica* erythrocytes (peak 2) from: A) a female individual (ID 6); B) a male individual (ID 17). In the inset table, information about the mean fluorescence of both sample and standard G_1 peaks is given, as well as, the DNA index (DI), genome size (pg/2C) and CV value (%).

Table 1
Genome size estimations of male and female individuals of *Pygoscelis antarctica* and *P. papua*. The results are presented as mean and standard deviation of the mean (SD). Also, for each species, the genome size difference between male and female estimates are provided. In brackets the mean genome size of each sex is given, if only the samples analysed using molecular sexing are considered. Different letters reveal statistically significant differences at $P < 0.05$ after Tukey test.

Species	Genome size (pg/2C) of males (mean ± SD)	Genome size (pg/2C) of females (mean ± SD)	Genome size difference (%)
Pygoscelis antarctica	2.99 ± 0.04[a] (2.98)	2.91 ± 0.03[b] (2.95)	2.6
Pygoscelis papua	2.98 ± 0.04[a] (2.97)	2.91 ± 0.03[b] (2.93)	2.3

3.3. Molecular sexing

The results of molecular sexing are given in Table 2. Using the PL/PR primers, it was possible to unequivocally determine the sex of the subset of samples selected for molecular sexing. Males due to their homogametic karyotype (ZZ), were characterized by a single band on the agarose gel corresponding to a single amplified fragment with approximately 276 bp, the *CHD1Z* gene that is amplified by the PL and PR primer pair. In the case of females, the heterogametic karyotype (ZW) led to the presence of two bands on the gel at approximately 276 bp and 294 bp, corresponding to the *CHD1Z* and *CHD1W* genes, respectively.

In Chinstrap penguins, the majority of the 22 individuals tested were males (81.8%), with only 18.2% of the individuals being identified as females. In Gentoo penguins, 60% of the 10 individuals tested were identified as males, whereas the remainder were females (40%).

3.4. Comparison between the sexing methods

When compared with the results of molecular sexing, only in 50.0% of all individuals (both species) did the sex discriminant functions of Polito et al. (2012) enable the correct assignment of sex (Table 2). Even so, these published discriminant functions did a much better job of correctly assigning sex in *P. papua* (90.0% correct) relative to *P. antarctica* (31.8% correct) based on their bill measurements. The poor success of discriminant functions in *P. antarctica* was due to the high number of males incorrectly classified as females (Table 2). In contrast, the determination of the sex by means of flow cytometry was equivalent to that of molecular sexing in 84.4% of the cases (*P. antarctica* = 86.4%; *P. papua* = 80.0%; Table 2). When flow cytometric sexing was compared with morphological sexing across all samples, similar sex assignment was observed in 52.6% of the cases (*P. antarctica* = 44.8%; *P. papua* = 60.7%). The agreement between these two methods was slightly improved (53.1%) when examining only those individuals that were sub-sampled for molecular sexing (*P. antarctica* = 45.5%; *P. papua* = 70.0%).

Following morphological sexing, males presented a larger bill length (6.3% larger in *P. papua* and 6.9% in *P. antarctica*) and bill depth (10.2% larger in *P. papua* and 5.7% in *P. antarctica*) than females (Table 3). Despite the fact that the sex of only half (approximately) of the individuals was correctly assigned, a similar tendency was observed after molecular and flow cytometric sexing, with males presenting 7 to 9% larger bill lengths and 6 to 7% larger bill depths, irrespective of the technique. The only exceptions were observed for *P. antarctica* after molecular sexing, as males presented similar values of bill length and smaller bill depth than females. Also, in *P. papua*, after flow cytometric sexing, males were only 1.6% larger than females for bill length and 0.6% larger for bill depth.

4. Discussion

For many decades, there has been a strong scientific interest in developing effective new tools for sex identification in bird species, especially when sexing individuals in populations of monomorphic species is important in behavioural and ecological studies (e.g. Catry et al., 2005; Cucco et al., 1999; Phillips et al., 2004; Xavier et al., 2003). In the beginning of the 1990s, flow cytometry started to be explored in other applications besides its use in clinical studies and immunology (Shapiro, 2007). One of the applications that was envisaged was the use of flow cytometry to determine the gender of birds (Nakamura et al., 1990). The development of a rapid, cheap and effective method to determine the sex of bird species with monomorphic plumage was particularly important, opening the possibility to estimate the sex in the early stages of bird development, when it is in many cases difficult to determine the sex.

Indeed, flow cytometry was used for gender estimation of numerous bird species (Cavallo et al., 1997; Cucco et al., 1999; Nakamura et al., 1990), its success being dependent on how different was the genome size between males and females (0.4–7% difference in genome size in birds), as a result of the differences in the size between the W and Z chromosomes. Considering the advantages of flow cytometry it was surprising that only a few papers have subsequently been published until now (e.g. Cucco et al., 1999; De Vita et al., 1994; Nakamura et al., 1990; Tiersch et al., 1991; Underwood et al., 2002). Despite the successful use of flow cytometry for bird sexing, a possible explanation for the lack of more studies may have been the development of DNA-based methods for the determination of the sex of an individual (reviewed in Ellegren and Sheldon, 1997a). In the review of Ellegren and Sheldon (1997a), the authors were highly critical of the use of flow cytometry for such purposes, and highlighted the fact that DNA-based methods related with the *CHD* genes were the best solution. Such criticism led to few future applications of flow cytometry in bird sexing (Ellegren and Sheldon, 1997b; Redelman et al., 1997). Indeed, to our knowledge, the last known paper where flow cytometry was used for estimating the sex of a bird species is the work of Underwood et al. (2002).

Our study is the first to use this technique applied to sex identification in penguins. Previously, some studies have attempted to develop reliable sex discriminant functions based on morphological data (Amat et al., 1993; Polito et al., 2012; Valenzuela-Guerra et al., 2013). Indeed, DNA-based molecular techniques are considered to be more reliable than morphological data (Hart et al., 2009), yet the discriminant function approach is considered a quick, minimally invasive and cost-effective method of sex classification (Dechaume-Moncharmont et al., 2011). Polito et al. (2012) developed discriminant functions based on bill length and bill depth for three penguin species collected at the Admiralty Bay, King George Island, Antarctica, and obtained a success rate ranging from 83.2% in Gentoo penguins to 96.7% in Chinstrap penguins. We found that applying these same discriminant functions to our dataset resulted in differing levels of success between species. Gentoo penguins had a success rate of 90.0% while successful sex assignment in Chinstrap penguins was only 31.8%. The lower level of success for discriminant functions in Chinstrap penguins may be due in part to morphological variation between King George Island and Livingston Island as has been found in previous studies of *Pygoscelis* penguins (Polito et al., 2012; Valenzuela-Guerra et al., 2013). If this was the case, we may have achieved better success for both species if we were able to calculate discriminant function equations based on data obtained from penguins of Livingston Island. Unfortunately, the generally low sample size of individuals sub-sampled for molecular sexing and especially the lack of many female Chinstrap penguins prohibited our ability to develop a robust, location-specific discriminant function. Another possible explanation for the poor success of discriminant functions in

Table 2
Genome size estimations of *Pygoscelis antarctica* and *P. papua* individuals, and comparison between the three methods used to discriminate the sex of each individual. The results are given as mean, standard deviation of the mean (SD), coefficient of variation of the mean (CV, %) and number of replicates (n).

Species/Individual	Genome size (pg/2C)				FCM sexing	Morphological sexing	Molecular sexing
	Mean	SD	CV (%)	n			
P. antarctica 1	2.98	0.03	1.03	3	M	F	M
P. antarctica 2	2.96	0.01	0.34	2	M	F	–
P. antarctica 3	2.87	0.02	0.79	2	F	F	M
P. antarctica 4	2.91	0.06	2.16	2	F	F	–
P. antarctica 5	2.91	0.04	1.28	3	F	F	–
P. antarctica 6	2.94	0.02	0.80	3	F	F	–
P. antarctica 7	3.02	0.03	0.85	3	M	F	–
P. antarctica 8	2.96	0.02	0.69	3	M	F	M
P. antarctica 9	3.06	0.07	2.13	3	M	F	–
P. antarctica 10	2.96	0.01	0.43	3	M	F	M
P. antarctica 11	2.96	0.03	1.02	3	M	F	M
P. antarctica 12	2.96	0.02	0.79	3	M	M	M
P. antarctica 13	3.02	0.00	0.14	2	M	F	M
P. antarctica 14	2.99	0.06	2.03	3	M	F	M
P. antarctica 15	3.02	0.07	2.41	2	M	M	M
P. antarctica 16	2.97	0.05	1.53	3	M	M	M
P. antarctica 17	2.98	0.01	0.28	3	M	F	M
P. antarctica 18	2.91	0.01	0.44	3	F	F	F
P. antarctica 19	3.00	0.03	0.88	3	M	F	–
P. antarctica 20	3.00	0.02	0.82	3	M	M	M
P. antarctica 21	2.99	0.03	0.98	3	M	M	M
P. antarctica 22	3.12	0.00	0.15	2	M	F	M
P. antarctica 23	3.02	0.02	0.58	3	M	F	M
P. antarctica 24	2.95	–	–	1	M	F	M
P. antarctica 25	2.94	0.00	0.09	3	F	F	F
P. antarctica 26	2.98	0.02	0.53	3	M	M	F
P. antarctica 27	2.97	0.01	0.27	3	M	M	F
P. antarctica 28	3.01	0.01	0.37	3	M	F	M
P. antarctica 29	2.95	–	–	1	M	F	M
P. papua 1	2.97	0.04	1.25	3	M	M	M
P. papua 2	3.07	0.01	0.35	2	M	M	–
P. papua 3	2.91	0.04	1.29	3	F	F	–
P. papua 4	2.96	0.05	1.79	3	M	M	M
P. papua 5	3.04	0.05	1.76	3	M	M	–
P. papua 6	2.95	0.00	0.12	3	M	F	M
P. papua 7	2.97	0.02	0.70	3	M	M	M
P. papua 8	2.95	0.01	0.49	3	M	F	–
P. papua 9	3.01	–	–	1	M	M	M
P. papua 10	2.93	0.03	1.08	3	F	F	F
P. papua 11	2.95	–	–	1	M	M	–
P. papua 12	2.91	0.00	0.14	3	F	F	F
P. papua 13	2.94	0.01	0.48	3	F	M	M
P. papua 14	2.94	0.02	0.64	3	F	F	–
P. papua 15	2.91	0.03	1.04	2	F	M	–
P. papua 16	3.03	–	–	1	M	F	F
P. papua 17	2.97	0.01	0.37	2	M	F	–
P. papua 18	2.93	0.06	2.09	2	F	F	–
P. papua 19	2.96	–	–	1	M	F	–
P. papua 20	3.01	–	–	1	M	F	–
P. papua 21	3.01	0.01	0.45	2	M	F	–
P. papua 22	2.92	0.01	0.33	3	F	F	–
P. papua 23	2.93	0.02	0.82	2	F	F	–
P. papua 24	2.85	0.04	1.27	2	F	F	F
P. papua 25	2.89	0.02	0.60	2	F	M	–
P. papua 26	2.97	0.05	1.60	2	M	M	–
P. papua 27	2.96	0.02	0.71	3	M	F	–
P. papua 28	2.98	0.02	0.72	2	M	M	–

Chinstrap penguins is variable age structure in our random sample of adults. Mínguez et al. (2001) found that first-time breeding adult Chinstrap penguins have smaller bills than older, more experienced breeders. We could not confirm the breeding status of the adults sampled in our study and thus the inclusion of young, non- or first-time breeding males with smaller bills may have led to their misclassification as females using the discriminant function of Polito et al. (2012).

In order to explore the possibility of using flow cytometric methods for sexing penguins, the genome size of all individuals was estimated and the results were compared with sexing obtained using DNA-based methods. Our results show that in approximately 85% of the cases, the determination of the sex by means of flow cytometry was equivalent to that of molecular sexing. Such a comparison enabled us to observe two clearly distinct genome size classes, with males presenting the highest values (2.99 pg/2C on average) and females the lowest ones (2.91 pg/2C). Furthermore, our results were highly reproducible and showed very low coefficients of variation, and no difficulties were observed when individuals were at either end of the genome size scale. Still, in

Table 3
Measurements of bill length and bill depth of *Pygoscelis antarctica* and *P. papua* according with the sex determination following each method. Values are given as mean and standard deviation of the mean.

Species	Sex	Bill length		Bill depth	
		Mean	SD	Mean	SD
Morphological sexing					
Pygoscelis antarctica	F	47.1	2.6	17.1	1.4
	M	50.3	2.1	18.1	1.2
Pygoscelis papua	F	45.7	1.6	15.2	0.8
	M	48.6	1.4	16.7	0.5
Flow cytometric sexing					
Pygoscelis antarctica	F	45.4	0.8	16.5	1.2
	M	48.5	2.8	17.6	1.4
Pygoscelis papua	F	46.5	1.8	15.8	0.7
	M	47.3	2.2	15.9	1.3
Molecular sexing					
Pygoscelis antarctica	F	48.1	3.3	18.2	0.6
	M	48.2	2.8	17.6	1.5
Pygoscelis papua	F	44.8	1.2	15.3	1.2
	M	48.7	1.0	16.4	0.6

several individuals, due to the low genome size difference between male and female individuals (only 2%, approximately), intermediate values (2.94–2.95 pg/2C) were observed, which hindered a total certainty of sex assignment in these cases. The occurrence of a range of DNA values for each gender has been detected before and may be attributed to chromosomal polymorphisms and repeated DNA sequences (Tiersch et al., 1991) or to subtle differences in the procedures and tests of individuals (Cucco et al., 1999).

When compared with DNA-based methods, both techniques require only a few microliters of whole blood, but flow cytometry is undoubtedly a less laborious and time-consuming technique (the analysis of each sample can be completed in 5 min) than the PCR-based procedures. Therefore, in case a flow cytometer is readily available, flow cytometry can be regarded as a much cheaper approach, considering the low volumes of nuclear isolation buffers (that can be prepared by hand) and fluorochrome that are needed to prepare each sample. The only theoretical limitation of flow cytometry is related to those cases where the genome size difference between males and females is not high enough to be resolved with certainty (e.g. De Vita et al., 1994), or in situations where there is a continuous range of DNA values (e.g. Underwood et al., 2002), as was the case of the two penguin species studied here. In both these situations, the accuracy of the genome size estimations is much more dependent on the quality of the samples and of the analyses. As PCR is not constrained by these limitations it can be used preferentially to flow cytometry in those cases, and, as stated above, has been the preferred method used by ornithologists (e.g. Griffiths et al., 1998; Martín et al., 2000; Zhang et al., 2013).

Due to these drawbacks, flow cytometry cannot be considered in all cases as a substitute for other sex identification tools, especially for sex-specific DNA probes. Still, in the particular case of Gentoo and Chinstrap penguins, our study shows that flow cytometry can be considered an effective and very rapid alternative to morphological sexing, being independent of the geographical origin of the sampled individuals or of age structure, as long as DNA-based methods are used to confirm the sex of those samples that present intermediate values of genome size. Therefore, with the current study it is shown that flow cytometry together with DNA-based methods in some targeted samples can be used effectively in the estimation of the gender of two penguin species, with the potential to be explored further in other penguin species.

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