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Genetic and morphological sex identification methods reveal a male-biased sex-ratio in the Ivory Gull *Pagophila eburnea*

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Concise and informative title: Sex identification in Ivory Gull

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Abstract

Sex identification of birds is relevant to studies of evolutionary biology and ecology and is often a central issue for the management and conservation of populations. The Ivory Gull *Pagophila eburnea* (Phipps, 1774) is a rare high-Arctic species whose main habitat is sea ice throughout the year. This species is currently listed Near Threatened by the IUCN, because populations have drastically declined in some part of the species distribution in the recent past. Here we tested molecular sexing methods with different types of samples. Molecular sexing appeared very efficient with DNA extracted from muscle, blood, and buccal swabs, both for adults and young chicks. We also performed morphological analyzes to characterize sexual size dimorphism in Ivory Gulls sampled in three distinct regions: Greenland, Svalbard and Russia. Males were larger than females for all morphometric measurements, with little overlap between sexes. Discriminant analysis based on six morphometric variables correctly classified ~95% of the individuals, even when using two variables only, *i.e.*, gonys height and skull length. Therefore, both molecular and biometric methods are useful for sexing Ivory Gulls. Interestingly, our results indicate a male-biased sex-ratio across all Ivory Gull populations studied, including two samples of offspring (67.8 % males).

Keywords: Molecular sexing; Morphological sexing; Sexual dimorphism; Noninvasive sampling; Buccal swab; Arctic.

Introduction

The ability to identify the sex of birds is important for management and conservation issues, and is relevant for many aspects of population biology, behavior research, and ecology. Although morphological differences can be marked between sexes in bird species, sexual dimorphism is often subtle in appearance or escape the human vision (Endler and Mielke 2005). Distinguishing males from females can be problematic in the field and methods have been developed to overcome this issue, including anatomical investigations (Miller et al. 2007), vocalization analyses (Krull et al. 2012), sex-specific behavior observations (Bosman et al. 2012; Yoon et al. 2013), and molecular techniques (Griffiths et al. 1998; Fridolfsson and Ellegren 1999).

Molecular sexing generally provides the best accuracy, but is not completely error-free because of the occurrence of allelic dropouts, when one allele of a heterozygous individual is not amplified during a positive PCR, usually the non-amplification of the W for the heterogametic female (WZ) that is then sexed as male (ZZ) (Arnold et al. 2003; Robertson and Gemmell 2006; Casey et al. 2009). Blood or plucked feather samples are usually used to extract DNA for sex identification in bird species with no apparent sexual dimorphism. There is however an on-going discussion about the use of these two methods in a conservation context (Lefort et al. 2015) because these DNA sampling methods are harmful and may have significant negative effects on structural integrity, fitness or behavior of organisms (O'Reilly and Wingfield 2001; Sheldon et al. 2008; Voss et al. 2010; McDonald and Griffith 2011). Moreover, these methods require proper training and, in most countries, a specific permit, which implies financial costs and delays for processing the samples.

Buccal cells collected using cotton swabs provide an alternative and less invasive source of DNA for sexing birds. Buccal swabs are now regularly used for population genetics in a number of species: amphibians (Pidancier et al. 2003; Broquet et al. 2007a; Gallardo et al. 2012), fish (Reid et al. 2012) and mammals (Corthals et al. 2015). Buccal swabs have lately been used in bird studies (e.g., Bush et al. 2005; Handel et al. 2006; Brubaker et al. 2011; Yannic et al. 2011), and a few studies have demonstrated the reliability of this sampling method for bird sex identification (Arima and Ohnishi 2006; Handel et al. 2006; Wellbrock et al. 2012; Dawson et al. 2015). Finally, a strictly non-invasive approach would be to sex birds using DNA extracted from shed feathers. Shed feathers yield DNA that is both less concentrated and more degraded (e.g., Yannic et al. 2011), and its applicability for sexing has yet to be assessed (but see Hogan et al. 2008).

Sexing based on morphological characteristics can also be a simple and efficient alternative method for sex identification for apparently monomorphic bird species (Dechaume-Moncharmont et al. 2011). In Laridae species, previous studies have shown that males are significantly larger than females and discriminant functions based upon external measurements can be used to sex individuals with a good reliability albeit differences can be small (Croxall 1995; Bosch 1996; Mawhinney and Diamond 1999; Chochi et al. 2002; Arizaga et al. 2008; Galarza et al. 2008; Aguirre et al. 2009; Herring et al. 2010; Bosman et al. 2012). With this approach, reliable discriminant functions must first be obtained through specific pilot-studies.

The Ivory Gull *Pagophila eburnea* is a medium-sized gull, endemic to the Arctic where it lives almost exclusively in sea-ice habitats outside the breeding season (Gilg et al. 2010). The species is listed as near-threatened by the International Union for

105 Conservation of Nature (IUCN) red list (BirdLife International 2012). Breeding
106 populations are found in the Canadian high Arctic, Svalbard (Norway), Greenland and
107 Russia and an international circumpolar 'Conservation Strategy and Action Plan' has
108 been presented by leading seabird experts from Arctic countries to gain more insight into
109 how this bird responds to increasing threats from disappearance of sea ice habitat, natural
110 resource exploration and increased contaminant loads (Gilchrist et al. 2008). An accurate
111 method to identify the sex of individuals would be useful to understand better the life
112 history and population dynamics of this species. Determining the sex of Ivory Gull from
113 visual observation in the field is difficult since there are no obvious visible characters that
114 differentiate between males and females.

115 In this study, we aimed to: 1) assess whether a molecular approach is applicable to
116 determine the sex of adult Ivory Gulls and nestlings (aged between 0 and 7 days); 2)
117 determine if buccal swabs and shed feathers are useful DNA sources in this regard, by
118 comparison with other sampling methods; 3) quantify morphological differences between
119 male and female Ivory Gulls and derive a discriminant function using the measurements
120 to facilitate sexing in the hand; and 4) test if morphology-based methods to identify sex in
121 one population could also be used for other populations with possibly different ecological
122 conditions.

123

124 **Methods**

125 **Study sites and sampling**

126 Ivory Gulls were sampled in summers 2006 to 2012 during the breeding season (late June
127 to early August). Sampling locations were distributed across the entire breeding range of

128 the species, including the Canadian Arctic Archipelago, north-eastern Greenland,
129 Svalbard Archipelago, Franz Josef Land Archipelago, Severnaya Zemlya Archipelago
130 and Kara Sea Islands (Table 1). We collected samples either in breeding colonies or
131 opportunistically near two military stations where Ivory Gulls are attracted by food
132 remains (i.e., Station Nord, Greenland and Alert, Canada). Trapping and handling
133 methods are described in Yannic et al. (2011; in press). Three nondestructive DNA
134 sampling methods (buccal swabs, plucked feathers and blood) and a noninvasive
135 sampling method (shed feathers) were used. Pieces of tissue were also opportunistically
136 collected on dead birds. Sampling methods broke down as follows: blood sampling
137 (*n*=82), buccal swabbing (*n*=184), collection of plucked feathers and shed feathers found
138 in nests and in the colonies (*n*=79), and sampling of tissue (muscle) on dead nestlings
139 (n=34, Yannic et al. 2014). In total, we collected samples from 307 adult birds and 72
140 nestlings (Table 1).
141
142 **Molecular sex identification**
143 Genomic DNA from all individuals was extracted from shed and plucked feathers, tissue,
144 blood or buccal swabs following protocols described in Yannic *et al.* (2011). Briefly,
145 DNA was extracted with Qiagen DNeasy Tissue Kit or the BioSprint robotic workstation
146 (Qiagen), eluted in a 200 µl Qiagen Buffer AE and stored at -20 °C. Birds were sexed
147 following two alternative protocols (Table 1) that targeted different fragments of the
148 conserved chromo-helicase-DNA binding protein (CHD) gene of the W and Z sex
149 chromosomes, using the P8/P2 (Griffiths et al. 1998) or the 2550F/2718R (Fridolfsson
150 and Ellegren 1999) primer sets.

151

152 *Sexing using the 2550F/2718R primer set*

153 PCR amplifications were carried out for 55 samples in 10µl containing 1 x PCR buffer
154 (QIAgen, Germantown, MD, USA), 2.5 mM of MgCl2, 0.2 mM dNTPs, 0.2 µM of
155 primers 2550F and 2718R (Fridolfsson and Ellegren 1999) and of 0.5 U *Taq* polymerase
156 (QIAgen, Germantown, MD, USA). PCR conditions were as follow: initial denaturation
157 at 94°C for 5 min; 30 cycles of 94°C for 30 sec (denaturation), 55°C for 30 s (annealing),
158 72°C for 1 min (elongation); and final elongation at 72°C for 10 min. PCR products were
159 separated in 2% agarose gels, run in standard TBE buffer, and visualized by ethidium
160 bromide staining. In Ivory Gull, PCR with the primer set 2550F/2718R yields a product
161 of 660 base pairs (bp) for the Z chromosome and a product of 420 bp for the W
162 chromosome.

163 We ensured the sex specificity of the 2550F/2718R primer pair by amplifying and
164 sequencing 6 birds (4 females and 2 males). PCR products were cloned using the TOPO
165 TA cloning kit (Life Technologies). Eight clones per sample were then amplified using
166 the above-described protocol. PCR products were separated in 2% agarose gels, run in
167 standard TBE buffer, and visualized by ethidium bromide staining. Two positive PCRs
168 products per sample (one Z and one W copy for females, and two Z copies for males)
169 were sequenced in both directions, analyzed on an ABI PRISM 3130XL genetic analyzer
170 (Applied Biosystems Foster City, CA, USA), aligned with MEGA 6 (Tamura et al. 2013),
171 and edited in SEAVIEW (Gouy et al. 2010). Sequences were then compared to the BLAST
172 Assembled Genomes database using the blastn algorithm.

173

Sexing using the P8/P2 primer set

Molecular sexing using the P8/P2 primer set (Griffiths et al. 1998) was performed by Wildlife Genetics International Inc. (D. Paetkau; Nelson, BC, Canada) following an optimized amplification procedure described in Paetkau et al. (1998), with a final concentration of 2 nM $MgCl_2$ and 0,640 µM of each primer. In Ivory Gull, PCR with the primer set P8/P2 yields a product of 282 bp for the Z chromosome and a product of 287 bp for the W chromosome. Amplification products were run on an ABI PRISM 3100 (Applied Biosystems) automated DNA sequencer. Alleles were scored with GENEMAPPER 4.1 (Applied Biosystems).

Reliability of molecular sexing

With both methods the sex of an individual was identified only if amplification yielded a clear, strong pattern. Weak signals, *e.g.*, determined by peak height ("relative fluorescence units") were considered as failed amplifications. With this approach, each successful amplification is associated with a sex identification. We thus estimated the performance of molecular sexing by estimating a rate of amplification success (number of successful PCR = number of amplifications leading to a sex identification) and a rate of error (number of successful PCR yielding the wrong sex). The reliability of the molecular sex identification in Ivory Gull was evaluated using a multi PCR approach for a subset of 139 birds from different sources of DNA (swab, feather or tissue) and for different age classes (adult versus juvenile). With this procedure each sample was amplified at least four times (and up to 7 times) using the P8/P2 primer set. The sex obtained for each individual was compared across repetitions in order to estimate the error rate associated

with molecular sexing. Finally, fifteen samples were amplified both with the P8/P2 primer set and the 2550F/2718R primer set to check the consistency between the two protocols.

Morphological measurements

To evaluate the reliability of morphological measurements for sexing adult Ivory Gulls, we used birds sampled in the north easternmost corner of Greenland, Station Nord (81°35'N, 16°39'W). Station Nord is a military station located on a coastal terrace, at about 3 km from a breeding colony (Gilg et al. 2009). Field observations and satellite tracking suggest that non-breeding adults from nearby colonies also visit or stay in colonies during the breeding season (O. Gilg and A. Aebischer, unpublished data). Thus, individuals analyzed here may be breeding birds from different colonies or non-breeding adult birds (*e.g.*, failed breeders) (Yannic et al. in press). For each bird, we measured skull (*i.e.*, total head: from the back of the head to the tip of the bill) and wing length (*i.e.*, from the elbow to the tip of the longest primary feathers) as well as gonys height (*i.e.*, bill depth at gonys). Standardized measurements were made with a digital caliper; while wing chord length was measured with a ruler and body mass was recorded using a Pesola spring scale (precision: 5 g). The sex obtained with the molecular approach (based on buccal swabs) was used as a reference for analyzing these data. For most birds of Greenland, we also measured tarsus and bill lengths, but since they did not contribute much in our preliminary analyses (data not shown) and because they were not systematically measured across the species range, we did not include them in the following analyses.

220

221 **Statistical analysis of morphological measurements**

222 Two-sample Wilcoxon rank sum tests were used to analyze the sexual dimorphism in
223 birds. All tests were performed in R version 3.1.2 (R Development Core Team 2014).

224 In order to identify combinations of morphological variables that would enable
225 sex identification, we performed linear discriminant function analyses based on six
226 morphological variables, using the lda function implemented in the "MASS*"* package for
227 R (Venables and Ripley 2002), with the prior probabilities of class membership set to 0.5.
228 Potential morphologic outliers were detected by measuring robust Mahalanobis distances
229 using the chisq.plot function from the "mvoutlier" R package (Filzmoser and
230 Gschwandtner 2014). The effect of outliers was assessed by removing such samples from
231 the data set, and estimating sex identification success in new discriminant analyses. We
232 performed forward/backward variable selection to identify the combination of variable
233 that allow sex identification with the best accuracy. The variable selection was performed
234 using the stepclass function (in both backward and forward direction with an
235 improvement of performance measure set to 5%) and using the minimization of Wilk's
236 lambda criterion (with a predefined significance level of 0.05), a frequently used
237 procedure in the bird literature (but see discussion in Dechaume-Moncharmont et al.
238 2011), as implemented in the "*klaR"* package in R.

239 We used three validation methods to estimate the proportion of correctly assigned
240 individuals by discriminant function analyses, that is resubstitution, leave-one-out cross-
241 validation (LOOCV), and repeated random sub-sampling cross-validation (RRSS). With
242 the resubstitution, the sex of each individual is predicted using the lda function obtained

243 from the complete data set. The resulting sex classification was then compared with the
244 sex identification obtained with the molecular sexing method. Using the LOOCV method,
245 the sex of an individual is predicted from the lda function obtained after this individual
246 has been removed from the data set (CV=TRUE in the lda R function). With the repeated
247 random sub-sampling cross-validation, the data set is randomly split into training and
248 validation subsamples. The training set (2/3 of the data) is used to compute the LDA
249 function that is then used to classify the remaining 1/3 of the individuals. The predictive
250 reliability of the LDA function is then assessed using the validation dataset. This
251 procedure was repeated 1000 times.

252 Finally, we assessed if the discriminate functions obtained in Greenland can also
253 be used in other populations across the species ranges, *i.e.*, Auga, Svalbard (n=17; Table
254 3) and Hayes Island, Franz Josef Land and Domashny, Severnaya Zemlya, Russia (n=32;
255 Table 4). Morphological differences between the three regions were investigated for
256 weight, wing and skull length and gonys height using two-way ANOVA in R with sex as
257 a co-factor. All results were considered significant at $P < 0.05$. Then we applied the
258 discriminant function obtained with the samples from the Greenland training set to
259 morphologically sex birds from Svalbard and Russia. The sex obtained thereby for each
260 individual was compared with molecular sex information.

261

262 **Results**

263 **Molecular sex identification**

264 Amplification with the 2550F/2718R primer pairs (Fridolfsson and Ellegren 1999)
265 produced either one or two bands, consistent with expectations for males and females,

respectively. Cloning and sequencing of 2550F/2718R PCR products confirmed the sex-specificity of the primer pair for Ivory Gull. Similarly, the P8/P2 primers produced amplicons of size 282 or 282/287 bp, as expected for male and female birds. Out of 139 samples that were repeatedly analyzed four to seven times, a single one produced contradictory sex identifications (this particular sample produced one male identification, one female identification, and six failed amplifications). The risk of error associated with molecular sexing is thus extremely low: only one error was detected out of 409 sex identifications (*i.e.*, sexing error rate < 0.0025 when using a single genotyping attempt). Moreover, the fifteen individuals that were tested with both methods produced consistent results (9 females and 6 males).

Overall, PCR amplifications based on blood were successful in 76 out of 82 samples (93% of amplification success, Fig. 1). We determined 54 males and 22 females. Using DNA from buccal swabs, a PCR product could be amplified in 160 out of 184 birds (90 % of success), revealing 112 males and 48 females. Using DNA from shed feathers, we determined the sex of 37 out of 79 birds (47%; 22 males and 15 females). Finally, sex identification based on tissue samples was successful in all samples (*n*=34; 20 males and 14 females). Overall we successfully sexed all nestlings using DNA from swabs (*n*=31) or from muscle (*n*=34). For adults (blood, swabs, and feathers combined), the overall success rate was 77% (87% using blood and swabs only). Overall, the genetic sex identification revealed that out of 307 samples successfully sexed, 208 were males (sex-ratio: 67.8% overall, 62.9% for juveniles, and 69.2% for adults).

289 **Morphological sex identification in Greenland**

290 Molecular sex identification was successfully obtained for 85 out of 105 adult Ivory Gulls
291 from northeastern Greenland (55 males and 30 females). Morphological information was
292 available for 77 of these birds (48 males and 26 females). Analyses showed that males
293 were significantly larger than females for all external morphological measures (Table 2;
294 $P < 0.001$ in all two-sample Wilcoxon rank sum tests), although with slightly overlapping
295 ranges.

296 Using all variables, the following discriminant equation was obtained:

297 $$D = -38.435 + 0.005 \times V_{Weight} \times 0.268 + V_{Wing} \times 0.136 + V_{Skull} \times 1.203 + V_{Gonys} \quad [1]$$

298 According to the full equation [1], a bird was classified as male when $D>0$ and as female
299 when $D<0$. Validation through the resubstitution method estimated that 94.6% of the
300 birds were sexed correctly (96.2% for females and 92.3% for males), whereas with the
301 LOOCV and RRSS cross-validation methods the proportions of correctly sexed adults
302 were 93.2% (92.3% for females and 93.8% for males), and 93.4% (93.6% for females and
303 93.2% for males), respectively (Table 2). For all misclassified individuals, *i.e.*, for which
304 molecular and morphological sexing differ, the sex was identified from four to seven
305 PCR repetitions.

306 Several potential morphological outliers were identified using the robust
307 Mahalanobis distances (two males and three females; all amplified four to seven times).
308 These morphological outliers had a slight effect in our analyses, because for four out of
309 five of them not located in the regions of morphological overlap between sexes.
310 Therefore, removing these individuals from the data set increased only slightly the
311 proportion of correctly sexed birds: resubstitution 95.6% (95.8% for females and 95.6%

for males), LOOCV 92.8% (91.7% for females and 93.3% for males), and RRSS 92.3% (91.1% for females and 93.1% for males).

The stepclass function for automated variable selection suggested a set of two variables was enough to accurately discriminate the sexes (skull length and gonys height). Stepwise variable selection led to the selection of the same two-variable set. Then, we used these two variables and performed the same analyses detailed above. The performance of this reduced dataset for sexing birds was very similar to the results obtained on the complete variable set (Table 2). Using all available data did not improve the proportion of correctly sexed birds, with a difference < 1% of individuals correctly classified between the original (*n*=6 measurements) and reduced (*n*=2 measurements) sets of variables. Using gonys and skull variables, the following reduced discriminant equation was obtained:

$$D = -32.101 + 0.171 \times V_{Skull} + 1.411 \times V_{Gonys} \qquad [2]$$

According to equation [2], a bird was classified as male when $D>0$ and female when $D<0$. All misclassified birds had D values between -0.876 and 0.057 for equation [1] and between -0.770 and 0.4807 for equation [2].

Comparison of populations

Molecular sex identification of the Ivory Gulls from Svalbard (n=17) and Russia (n=32) revealed that there were 11 males and 6 females in our sample from Svalbard (Table 3) and 26 males and 6 females in Russia (Table 4). Morphology comparisons showed that males were significantly larger than females for all measures in Svalbard (Table 3; $P <$

0.001 in all two-sample Wilcoxon tests) and for all measures except wing length in Russia (Table 4).

We tested whether the morphology-based sex identification equations obtained using birds from Greenland would yield correct results in samples from these new geographic areas (Tables 3 and 4). Morphological differences between the two sexes have been observed in all regions (Tables 2,3,4 and Figure 3). Interestingly, we found significant differences between the regions for gonys height and weight, but not for wing length and skull length (Figure 3). The absence of difference in wing morphology may be related to the constraint on the species' flight performance (Croxall 1995). There was no significant interaction between sex and region (Figure 3). As it turned out, the discriminant function (equation [1]) derived from Greenland provided a reliable way to identify the sex of the birds from Svalbard and Russia too. Using the four morphological variables, LDA function performed with Greenland adult Ivory Gulls as a training set led to the correct identification of sex for 100% of the (*n*=6) females and 100% of the (*n*=11) males in Svalbard and for 66.7% of the (*n*=6) females and 100% of the (*n*=26) males in Russia. This result may be explained by the low number of females (n=6 in both testing sets) and the significant difference in weight and gonys length between Ivory Gulls from different regions (Figure 3). It is worth noting, however, that the contribution of weight is low in comparison with gonys height in the discriminant function [1]. Using the reduced linear discriminant analyses function based on gonys height and skull length (equation [2]) led, however, to the correct identification of sex for 83.3% of the (*n*=6) females and 100% of the (*n*=26) males in Russia (Table 4).

357 **Discussion**

358 **Molecular sexing**

359 The PCR-based methods of Griffiths et al. (1998) and of Fridolfsson and Ellegren (1999)
360 were suitable for sex discrimination of Ivory Gulls, yielding unambiguous profiles for
361 males and females. Sequencing of PCR products, cross-validation of the two protocols
362 and multiple repeats of amplifications for a set of samples confirmed the sex-specificity
363 of the primer pairs and their reliability to identify sex in Ivory Gulls. Buccal swabs
364 appeared to be a reliable source of DNA for sexing adults and juveniles. The amount of
365 DNA (see extract concentrations for various sample types in Yannic et al. 2011) was
366 sufficient to successfully perform PCRs, even with buccal swabs sampled on nestlings in
367 the first few days after hatching (success rate: 100% in juveniles, 85% in adult birds). The
368 overall amplification success obtained using swabs in this study (87.0%, n=184 samples)
369 is comparable to that obtained for sex identification from buccal swabs in twelve wild
370 bird species (82.2%, n=107 samples; Arima and Ohnishi 2006). Wellbrock et al. (2012)
371 showed that sex identification based on buccal swabs matched the result of sex
372 identification based on blood samples in juvenile (98%) and adult (100%) Common
373 Swifts *Apus apus*. Therefore, buccal swabbing is a reliable source of DNA for sex
374 identification in Ivory Gull, as in other birds. By contrast, shed feathers proved to be a
375 poorer alternative, with an overall sex identification success rate below 50%. This result
376 is consistent with those obtained for microsatellites (Yannic et al. 2011). Moreover, with
377 this method the risk of genotyping errors becomes an important issue: non-amplification
378 of the W fragment in females could yield wrong sex identifications. The risk of such
379 allelic dropout increases with decreasing DNA quantity and quality, but one can control

380 for this problem by repeating the genotyping several times when working with low
381 quality samples such as shed feathers. In this study we obtained a very low error rate
382 (only one erroneous sex identification) even with shed feathers, but this low rate of error
383 is in part due to the fact that we considered only strong PCR products, discarding any
384 weak amplification signal. In conclusion, shed feathers can be used for molecular sexing,
385 but at a high cost and with low overall efficiency.
386
387 **Morphological sexing**
388 Our results show that Ivory Gulls breeding in north-eastern Greenland present sexual
389 differences in their external morphology (Table 1 and Fig. 2). These birds can be sexed
390 by a discriminant function using a combination of only two morphological measures
391 (gonys height and skull length; equation [2]) with reliability ~95% (Table 1). The
392 inclusion of other morphological measurements in our model (equation [1]) did not
393 improve sensibly the percentage of individuals correctly classified (Table 1). In addition,
394 the contribution of body weight to the discriminant function was very low (see equation
395 [1]). Therefore, the reduced discriminant function will prove useful for sexing Ivory Gull
396 in the field and could shorten handling time, thereby allowing researchers to release birds
397 quickly after a minimum of disturbance (Chochi et al. 2002). Note that this method is
398 only 95% accurate, meaning that it should be used only if this level of accuracy is
399 acceptable for the study under consideration (e.g. rapid sex assessment for choosing
400 which birds should be equipped with Argos probes). A higher (100%) accuracy will be
401 achieved using DNA, e.g. from buccal swabs.

Applying the discriminant function [1] based on birds from Greenland as a training set allowed us to accurately sex birds from other breeding colonies located in Svalbard (100% success; Table 3), and to a lesser extent in Russia (93.8% success; Table 4). Equation [2] allowed, however, to correctly sexing 96.8% of Russian birds (83.3% of females and 100% of males). This result suggests that the method could be widely applicable (again, to the extent where such accuracy levels are acceptable for a given application). However, some Laridae species show variation for morphological traits across their distribution range, or age-related differences (Palomares et al. 1997; Meissner 2007). In the case of Ivory Gull, a recent study found that the species is strikingly genetically homogeneous across its entire breeding range (Yannic et al. in press). Yet there could still be phenotypic differences between birds from different areas. Additional morphometric data from other Ivory Gull colonies will thus be useful to test further the morphological sexing method proposed here. At present we suggest that the method can be employed when one needs rapid sex identification and when accuracy does not need to be higher than 95%.

Sex ratio

This methodological study yielded an interesting, unexpected result: with 208 males and 99 females genetically sexed, the sex ratio appeared quite strongly male-biased over the entire study area (binomial test $p << 0.001$, there are 67.8 % males, *i.e*. 2 males for 1 female,). This bias could have several explanations, which we briefly discuss below.

The first possibility is that there is some error in the molecular sexing. As mentioned above, allelic dropouts during PCR could lead us to wrongly identify females

425 as males. However, several observations refute strongly this dropout hypothesis. First and
426 foremost, our repetition experiment for 139 individuals genotyped 4 to 7 times showed
427 that the risk of error was very weak (< 0.0025). This result is remarkably consistent with
428 previous experiences showing that only about 0.25% of female samples of low DNA
429 quality might be affected by non-amplification of the diagnostic W allele with the P2/P8
430 primer pair (D. Paetkau, pers. comm.). Second, a set of samples (*n*=15) have been
431 double-checked using the method described in Fridolfsson and Ellegren (1999), which
432 targets another portion of the CDH gene (and the sex-specificity of this universal primer
433 pair has been controlled by sequencing Z and W alleles). The two methods lead to the
434 exact same results, 6 males and 9 females. The male-biased adult sex ratio was also
435 observed for birds of Russia sexed with blood samples using the method described in
436 Fridolfsson and Ellegren (1999) (proportion of males > 80%; Table 1). This control is
437 particularly useful because with the PCR-based method of Fridolfsson and Ellegren
438 (1999) the W band is sensibly shorter than the Z band, contrary to the method of Griffiths
439 et al. (1998). Since shorter fragments have a tendency to amplify more easily than longer
440 ones (Broquet et al. 2007b), it is unlikely that allelic dropouts have led to losing the W
441 allele preferentially. In addition, we never observed a WW genotype with any of our two
442 methods (which should be obtained in case of random allelic dropouts due to low DNA
443 quality). Finally, all DNA sources produced a biased sex ratio (Fig. 1), and the most
444 error-prone samples (shed feathers) yielded less bias than others (22 males and 15
445 females).
446 Another non-biological cause of sex-ratio bias could be the higher probability of
447 capture for males than females (*e.g.*, if males visited more trapping sites and female

448 provide more maternal care on nests). This hypothesis could be plausible for adults. It is,
449 however, not compatible with our observations of sex ratio in nestlings (44 males and 26
450 females, binomial test p=0.02), an age class where there can be no bias regarding the
451 probability of capture of males versus females. This suggests quite strongly that the
452 pattern is real, although further estimates of offspring sex-ratio from a few more sites
453 would be a welcome addition, at least to assess whether our observations can be
454 generalized (in this study offspring were sampled from two main sites, both located in
455 Greenland).

456 There is a wealth of mechanisms that can affect the balance between male and
457 female numbers at the initial offspring stage (reviewed e.g. in Danchin et al. 2008; West
458 2009). The hypotheses that are, perhaps, most likely applicable for a bird like the Ivory
459 Gull are as follows:

460 1) Secondary bias due to mortality. Female-biased embryo mortality could result
461 in an excess of males. Feeding high in the Arctic marine food chain, the Ivory Gull is
462 exposed to high levels of contaminants (Hobson et al. 2002). Studies showed high levels
463 of organohalogen contaminants in Ivory Gulls - among the highest reported in Arctic
464 seabird species - in liver and fat (Fisk et al. 2001; Buckman et al. 2004) as well as in eggs
465 (Braune et al. 2007; Miljeteig et al. 2009; Lucia et al. 2015). High levels of
466 organochlorines are known to affect hatching sex ratio towards males in Lesser Black-
467 Backed Gull (Erikstad et al. 2009) and in Arctic Glaucous Gull (Erikstad et al. 2011), for
468 which lower levels of contaminants have be detected in comparison to Ivory Gull
469 (Miljeteig et al. 2009). Consequently, one may not exclude a possible role of
470 contaminants in the observed skew in sex ratio toward male offspring in Ivory Gull.

471 2) Sex allocation based on parental condition. Females in good maternal condition
472 could increase their maternal investment in offspring, which would benefit male offspring
473 more (in particular if male size is under natural or sexual selection). In such situations
474 females are selected to produce more sons. While this predicts variations in sex ratio
475 among clutches rather than an overall bias, other factors such as the supplemental feeding
476 provided by the presence of military stations in Northern Greenland may interact locally
477 with sex allocation. Another mechanism of sex allocation based on parental condition is
478 the adjustment of sex ratio according to mate attractiveness. Females could be under
479 selection to produce more sons when mated to an attractive male (reviewed in West and
480 Sheldon 2002). We know too little of the Ivory Gull mating system to refute or confirm
481 this hypothesis, but again this would explain variations in sex ratio among families rather
482 than a systematic bias.
483 3) Competition or cooperation among relatives. A system where females are
484 philopatric and compete for resources (or males cooperate for access to females) induces
485 selection for male-biased sex allocation (Danchin et al. 2008; West 2009).
486 4) Distortion in the genetic determination system. For instance, sex ratio distorters
487 located on sex chromosomes (or, potentially, in the cytoplasm) could influence the ratio
488 of males to females produced (Danchin et al. 2008; West 2009).
489 We have too little information to discuss these theoretical hypotheses more
490 precisely. This intriguing result deserves further investigations, starting with additional
491 estimates of sex-ratio in nestlings from different colonies, in order to identify the
492 processes that drive the bias of sex ratio in this species. Concerning the adults,
493 investigations of colony and nest attendance (e.g., pattern of incubation, nestling rearing)

would also bring additional information on the behavior of males and females during the breeding season and on a potential bias of probability of capture between males and females.

Conclusion

Working in the extremely harsh high-Arctic environments with threatened species imposes a sampling as nonintrusive as possible. Reducing manipulation and handling time limit the number of measurements that can be recorded. Although we only investigated a limited number of morphological variables (n=4), we observed that a reduced method (*i.e.*, using only two simple field measurements) allowed the sex identification of ~ 95% of the individuals. Moreover, we showed that molecular sexing from buccal swabs is accurate for sensitive bird species like the Ivory Gull, for which it is important to minimize any possible stress induced by manipulation, handling time and sampling of the birds. Therefore, we strongly recommend using buccal swabs for sampling DNA from birds, and especially nestlings, which can be more difficult to sample for blood.

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Table 1. Samples used for evaluating the feasibility of molecular sexing using the P8/P2 primer pair (Griffiths et al. 1998) or the 2550F/2718R primer pair (Fridolfsson and Ellegren 1999) in Ivory Gull. N: sample size, M and F: numbers of males and females successfully sexed by molecular amplification.

ID	Country	Site	Latitude	Longitude	Status	DNA source	N	M	F	Sex ratio (%M)	Method
#1	Greenland	Station Nord	81.60	-16.66	Adult	Swab	105	55	30	64.7%	2
#2			81.61	-16.49	Juvenile	Swab/Tissue	20	15	5	75.0%	2
#3		Amdrup Land	80.85	-14.63	Juvenile	Swab/Tissue	45	27	18	60.0%	2
#4	Norway	Svenskoya	78.72	26.63	Adult	Blood	9	5	4	55.6%	1
#5		Auga	78.50	21.74	Adult	Swab/Blood	18	12	6	66.7%	2
#6		Hübnerbreen	78.41	21.69	Adult	Swab	7	5	2	71.4%	1
#7		Freemanbreen	78.38	21.43	Adult	Swab/Feather	35	24	7	77.4%	1
#8	Russia	Hayes Island	80.61	57.96	Adult	Blood	7	4	1	80.0%	3
#9		Nagurskoje	80.72	48.22	Adult	Feather	4	0	0	-	1
#10		Rudolf Island	81.75	58.39	Adult	Feather	15	2	1	66.7%	1
#11		Eva-Liv Island	81.64	63.22	Adult	Feather	3	0	0	-	1
#12		Schmidt Island	81.04	90.76	Adult	Feather	12	4	0	100%	1
#13		Domashny Island	79.51	94.84	Adult	Swab/Feather/Blood	59	39	13	75.0%	2
#14		Komsomalets Island	80.77	91.05	Adult	Feather	5	1	1	50.0%	1
#15		Sukhaya River	80.77	96.75	Juvenile	Feather	7	2	3	40.0%	1
#16		Heiberg Islands	77.61	101.51	Adult	Feather	4	2	0	100%	1
#17	Canada	Seymour Island	76.80	-101.27	Adult	Feather	11	4	2	66.7%	1
#18		Ellesmere Island (Alert)	82.50	-62.33	Adult	Blood	13	7	6	53.8%	1
							379	208	99	67.8%	

[1] Samples sexed with the P8/P2 primer pair (Griffiths et al. 1998)

[2] Samples sexed with the P8/P2 primer pair (Griffiths et al. 1998) and/or the 2550F/2718R primer pair (Fridolfsson and Ellegren 1999)

[3] Samples sexed with the 2550F/2718R primer pair (Fridolfsson and Ellegren 1999)

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Table 2. Morphometric characteristics of Ivory Gull from northern Greenland.

				Validation[2]				
	Females (n=26)	Males (n=48)	Sexual dimorphism[1]	resubstitution			LOOCV	RRSS
Measure	mean±sd	mean±sd	*W*	Females	Males	Overall	Overall	Overall
Weight (g)	493±36	562±43	141***	84.6%	79.2%	81.1%	-	-
Wing (mm)	34.0±0.9	35.2±0.8	186.5***	73.1%	81.3%	78.4%	-	-
Skull (mm)	89.4±2.8	96.2±3.2	41.5***	92.3%	89.6%	90.5%	-	-
Gonys (mm)	10.8±0.5	12.1±0.5	38.5***	88.5%	91.7%	90.5%	-	-
Weight x Wing x Skull x Gonys	-	-	-	96.2%	93.8%	94.6%	93.2%	93.4%
Skull x Gonys	-	-	-	92.3%	95.8%	94.6%	93.2%	93.5%

n: denotes sample size for individuals genetically sexed – for each measure, mean and standard deviation [sd] are provided

[1] As assessed by two-tailed Wilcoxon Rank Sum tests. Asterisks indicate significant values for the estimators: *P<0.05, **P<0.01, ***P<0.001, ns=non-significant differences

[2] Based on linear discriminant analysis, using three validation methods: resubstitution, Leave-one-out cross-validation (LOOCV) and Repeated random sub-sampling (RRSS) cross-validation

Table 3. Morphometric characteristics of Ivory Gull from Svalbard.

	Females (n=6)	Males (n=11)	Sexual dimorphism[1]	% Individuals correctly classified[2]		
	mean±sd	mean±sd	*W*	Females	Males	Overall
Weight (g)	488±39	620±51	0.00***	100.0%	100.0%	100.0%
Wing (mm)	33.7±0.6	35.2±0.7	2.00***	100.0%	90.9%	94.1%
Skull (mm)	88.7±1.7	96.4±1.4	0.00***	100.0%	100.0%	100.0%
Gonys (mm)	11.2±0.5	12.5±0.4	1.50***	83.3%	100.0%	94.1%
Weight x Wing x Skull x Gonys[3]	-	-	-	100.0%	100.0%	100.0%
Skull x Gonys[3]	-	-	-	83.3%	100.0%	94.1%

n: denotes sample size for individuals genetically sexed – for each measure, average and standard deviation [sd] are provided

[1] As assessed by two-tailed Wilcoxon Rank Sum tests Asterisks indicate significant values for the estimators: *P<0.05, **P<0.01, ***P<0.001, ns=non-significant differences

[2] Based on linear discriminant analysis

[3] Analysis based on Equation [1] and Equation [2] with birds of Greenland as training set

Table 4. Morphometric characteristics of Ivory Gull from Russia.

	Females (n=6)	Males (n=26)	Sexual dimorphism*	% Individuals correctly classified		
	mean±sd	mean±sd	*W*	Females	Males	Overall
Weight (g)	560±60	625±43	31.50*	33.3%	96.2%	84.4%
Wing (mm)	34.9±1.4	35.3±1.0	74.00[ns]	0.0%	100.0%	81.3%
Skull (mm)	91.2±1.5	95.9±2.6	9.00***	66.7%	92.3%	87.5%
Gonys (mm)	11.1±0.5	12.5±0.5	1.50***	83.3%	100.0%	96.9%
Weight x Wing x Skull x Gonys	-	-	-	66.7%	100.0%	93.8%
Skull x Gonys	-	-	-	83.3%	100.0%	96.9%

n: denotes sample size for individuals genetically sexed with the 2550F/2718R primer pair (Fridolfsson and Ellegren 1999) – for each measure, mean and standard deviation [sd] are provided

[1] As assessed by two-tailed Wilcoxon Rank Sum tests. Asterisks indicate significant values for the estimators: *P<0.05, **P<0.01, ***P<0.001, ns=non-significant differences

[2] Based on linear discriminant analysis with birds of Greenland as training set

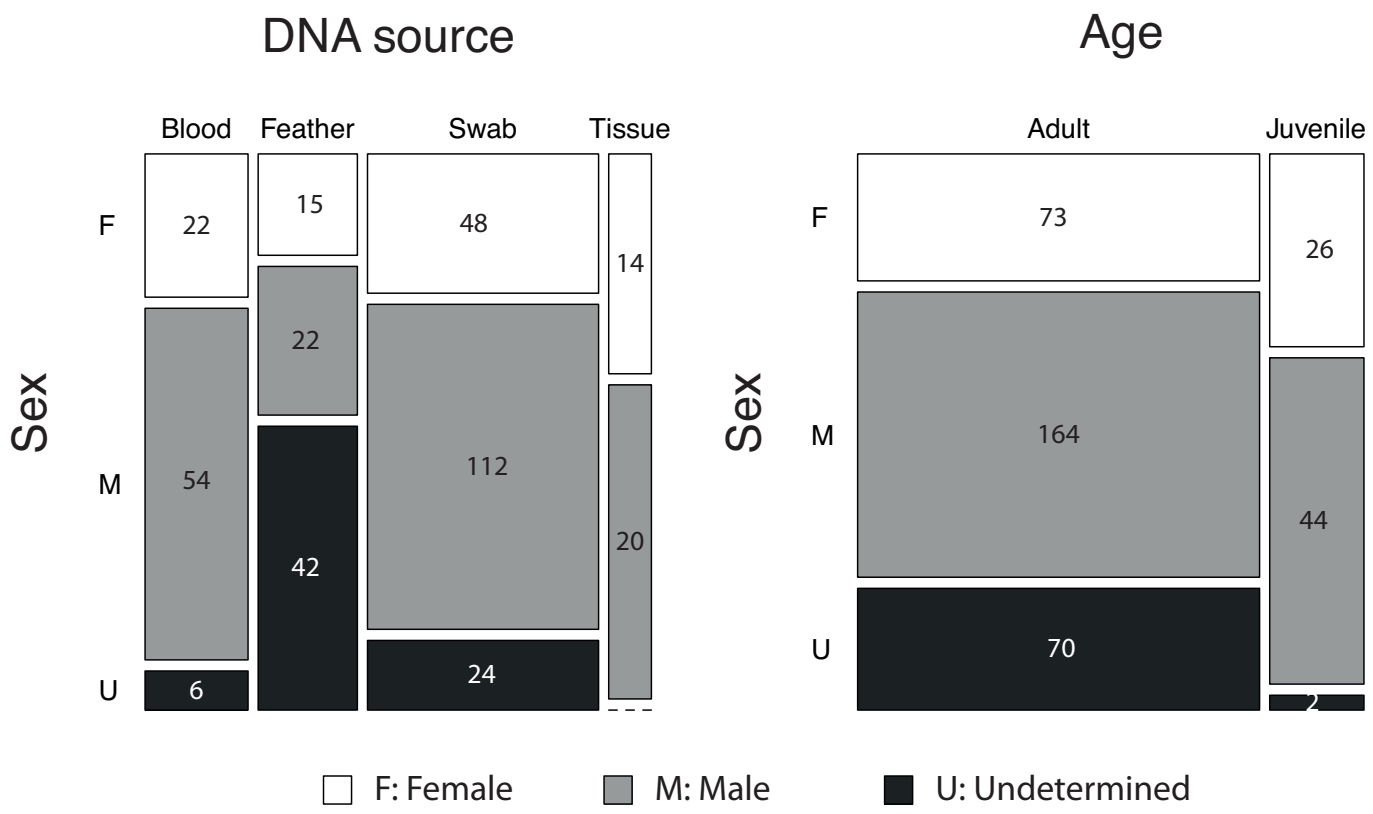
[3] Analysis based on Equation [1] and Equation [2] with birds of Greenland as training set

Figure captions

Figure 1. Results of molecular sex determination in Ivory Gull obtained either using the P8/P2 primer pair (Griffiths et al. 1998) or the 2550F/2718R primer pair (Fridolfsson and Ellegren 1999), broken down by DNA sources (left panel) and age class (right panel). The size of the boxes is proportional to the sample size.

Figure 2. Relationship between gonys height and skull length in Ivory Gull from North Greenland. The combination of these two measurements as cofactor in linear discriminant analyses correctly sexed ~ 95% of birds (Equation [2]). Solid line: classification boundary obtained by LDA. Grey diamond: mean and standard deviation for male and female gonys height and skull length, respectively.

Figure 3. Comparison of morphological measurements between sex of Ivory Gull in Greenland, Svalbard, and Russia**.** Significant morphological differences between the two sexes have been observed in all regions (all $P<0.001$) and only mean differences among regions are depicted here. Different letters over or above bars indicate significant pairwise differences among regions ($\alpha = 0.05$).


DNA source
Blood
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Age
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73
164
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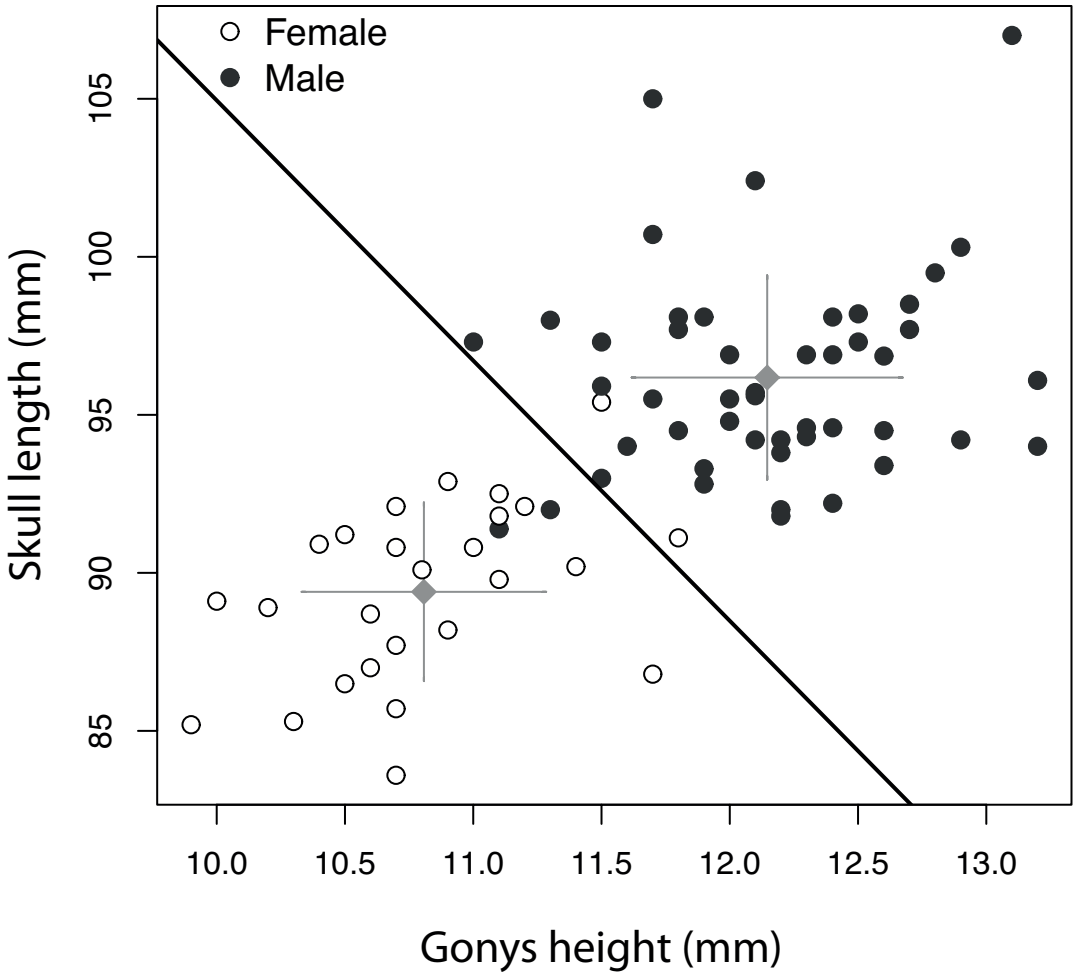
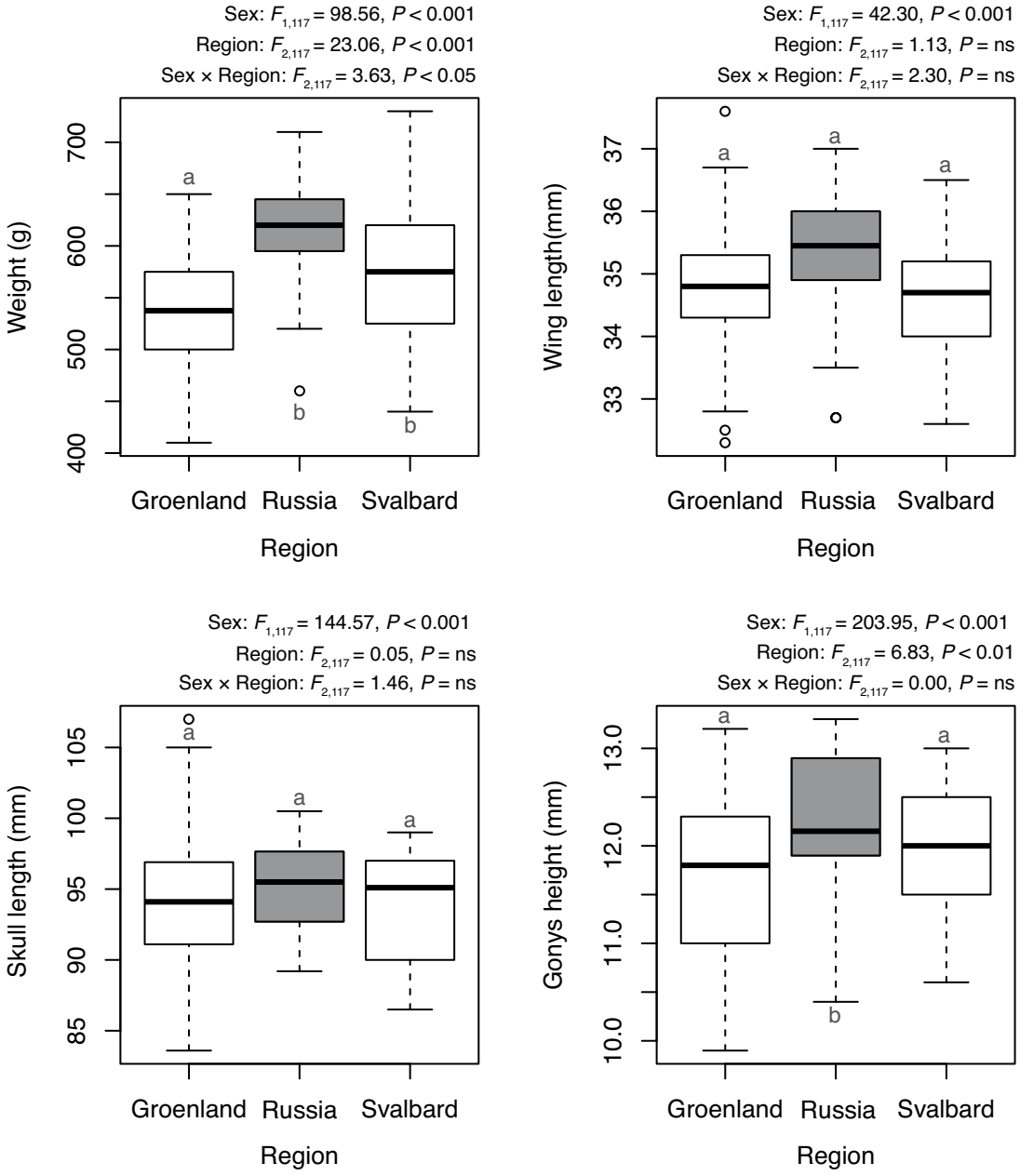
2
F: Female
M: Male
U: Undetermined


Female
Male
Skull length (mm)
105
100
95
90
85
10.0
10.5
11.0
11.5
12.0
12.5
13.0
Gonys height (mm)


Sex: $F_{1,117} = 98.56$, $P < 0.001$
Region: $F_{2,117} = 23.06$, $P < 0.001$
Sex × Region: $F_{2,117} = 3.63$, $P < 0.05$
Weight (g)
400
500
600
700
a
b
b
Groenland
Russia
Svalbard
Region
Sex: $F_{1,117} = 42.30$, $P < 0.001$
Region: $F_{2,117} = 1.13$, $P =$ ns
Sex × Region: $F_{2,117} = 2.30$, $P =$ ns
Wing length(mm)
33
34
35
36
37
a
a
a
Groenland
Russia
Svalbard
Region
Sex: $F_{1,117} = 144.57$, $P < 0.001$
Region: $F_{2,117} = 0.05$, $P =$ ns
Sex × Region: $F_{2,117} = 1.46$, $P =$ ns
Skull length (mm)
85
90
95
100
105
a
a
a
Groenland
Russia
Svalbard
Region
Sex: $F_{1,117} = 203.95$, $P < 0.001$
Region: $F_{2,117} = 6.83$, $P < 0.01$
Sex × Region: $F_{2,117} = 0.00$, $P =$ ns
Gonys height (mm)
10.0
11.0
12.0
13.0
a
b
a
Groenland
Russia
Svalbard
Region